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PATENT APPLICATION

HUMAN RECEPTOR PROTEINS; RELATED REAGENTS AND METHODS

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HUMAN RECEPTOR PROTEINS; RELATED REAGENTS AND METHODS

This filing is a continuation-in-part patent application, claiming benefit of U.S. Utility patent application Ser. No. 09/728,540, filed November 28, 2000, which claims benefit of U.S. Provisional Patent Application USSN 60/207,558, filed May 25, 2000, which claims priority to copending U.S. Patent Application 09/073,363, filed May 6, 1999, which claims benefit of the following Provisional Patent Applications: USSN 60/044,293, filed May 7, 1997; USSN 60/072,212, filed January 22, 1998; and USSN 60/076,947, filed March 5, 1998; all of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to compositions and methods for affecting mammalian physiology, including morphogenesis or immune system function. In particular, it provides nucleic acids, proteins, and antibodies which regulate development and/or the immune system. Diagnostic and therapeutic uses of these materials are also disclosed.

BACKGROUND OF THE INVENTION

Recombinant DNA technology refers generally to techniques of integrating genetic information from a donor source into vectors for subsequent processing, such as through introduction into a host, whereby the transferred genetic information is copied and/or expressed in the new environment. Commonly, the genetic information exists in the form of complementary DNA (cDNA) derived from messenger RNA (mRNA) coding for a desired protein product. The carrier is frequently a plasmid having the capacity to incorporate cDNA for later replication in a host and, in some cases, actually to control expression of the cDNA and thereby direct synthesis of the encoded product in the host.

For some time, it has been known that the mammalian immune response is based on a series of complex cellular interactions, called the "immune network". Recent research has provided new insights into the inner workings of this network. While it remains clear that much of the immune response does, in fact, revolve around the network-like interactions of lymphocytes, macrophages, granulocytes, and other cells, immunologists now generally hold the opinion that soluble proteins, known as lymphokines, cytokines, or monokines, play critical roles

in controlling these cellular interactions. Thus, there is considerable interest in the isolation, characterization, and mechanisms of action of cell modulatory factors, an understanding of which will lead to significant advancements in the diagnosis and therapy of numerous medical abnormalities, e.g., immune system disorders.

Lymphokines apparently mediate cellular activities in a variety of ways. They have been shown to support the proliferation, growth, and/or differentiation of pluripotential hematopoietic stem cells into vast numbers of progenitors comprising diverse cellular lineages which make up a complex immune system. Proper and balanced interactions between the cellular components are necessary for a healthy immune response. The different cellular lineages often respond in a different manner when lymphokines are administered in conjunction with other agents.

Cell lineages especially important to the immune response include two classes of lymphocytes: B-cells, which can produce and secrete immunoglobulins (proteins with the capability of recognizing and binding to foreign matter to effect its removal), and T-cells of various subsets that secrete lymphokines and induce or suppress the B-cells and various other cells (including other T-cells) making up the immune network. These lymphocytes interact with many other cell types.

Another important cell lineage is the mast cell (which has not been positively identified in all mammalian species), which is a granule-containing connective tissue cell located proximal to capillaries throughout the body. These cells are found in especially high concentrations in the lungs, skin, and gastrointestinal and genitourinary tracts. Mast cells play a central role in allergy-related disorders, particularly anaphylaxis as follows: when selected antigens crosslink one class of immunoglobulins bound to receptors on the mast cell surface, the mast cell degranulates and releases mediators, e.g., histamine, serotonin, heparin, and prostaglandins, which cause allergic reactions, e.g., anaphylaxis.

Research to better understand and treat various immune disorders has been hampered by the general inability to maintain cells of the immune system in vitro. Immunologists have discovered that culturing many of these cells can be accomplished through the use of T-cell and other cell supernatants, which contain various growth factors, including many of the lymphokines.

The interleukin-1 family of proteins includes the IL-1α, the IL-1β, the IL-1RA, and recently the IL-1γ (also designated Interferon-Gamma Inducing Factor, IGIF). This related

family of genes have been implicated in a broad range of biological functions. See Dinarello, FASEB J. 8, 1314 (1994); Dinarello, Blood 77, 1627 (1991); and Okamura, et al., Nature 378, 88 (1995).

In addition, various growth and regulatory factors exist which modulate morphogenetic development. This includes, e.g., the Toll ligands, which signal through binding to receptors which share structural, and mechanistic, features characteristic of the IL-1 receptors. See, e.g., Lemaitre, et al., Cell 86, 973 (1996); and Belvin and Anderson, Ann. Rev. Cell & Devel. Biol. 12, 393 (1996).

From the foregoing, it is evident that the discovery and development of new soluble proteins and their receptors, including ones similar to lymphokines, should contribute to new therapies for a wide range of degenerative or abnormal conditions which directly or indirectly involve development, differentiation, or function, e.g., of the immune system and/or hematopoietic cells. In particular, the discovery and understanding of novel receptors for lymphokine-like molecules which enhance or potentiate the beneficial activities of other lymphokines would be highly advantageous. The present invention provides new receptors for ligands exhibiting similarity to interleukin-1 like compositions and related compounds, and methods for their use.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic comparison of the protein architectures of Drosophila, Caenorabditis, and human TLRs, and their relationship to vertebrate IL-1 receptors and plant disease resistance proteins. Three Drosophila (Dm) TLRs (Toll, 18w, and the Mst ORF fragment) (Morisato and Anderson, Ann. Rev. Genet. 29, 371 (1995); Chiang and Beachy, Mech. Develop. 47, 225 (1994); Mitcham, et al., J. Biol. Chem. 271, 5777 (1996); and Eldon, et al., Develop. 120, 885 (1994)) are arrayed beside four complete (TLRs 1-4) and one partial (TLR5) human (Hu) receptors. Individual LRRs in the receptor ectodomains that are flagged by PRINTS (Attwood, et al., Nucleic Acids Res. 25, 212 (1997)) are explicitly noted by boxes; 'top' and 'bottom' Cys-rich clusters that flank the C- or N-terminal ends of LRR arrays are respectively drawn by opposed half-circles. The loss of the internal Cys-rich region in TLRs 1-5 largely accounts for their smaller ectodomains (558, 570, 690, and 652 aa, respectively) when compared to the 784 and 977 aa extensions of Toll and 18w. The incomplete chains of DmMst and HuTLR5 (about 519 and 153 aa ectodomains, respectively) are represented by dashed lines. The intracellular signaling module common to TLRs, IL-1-type receptors (IL-1Rs), the intracellular protein Myd88, and the tobacco disease resistance gene N product (DRgN) is indicated below the membrane. See, e.g., Hardiman, et al., Oncogene 13, 2467(1996); and Rock, et al., Proc. Nat'l Acad. Sci. USA 95, 588 (1998). Additional domains include the trio of Ig-like modules in IL-1Rs (disulfide-linked loops); the DRgN protein features an NTPase domain (box) and Myd88 has a death domain (black oval).

Figures 2A-2C show conserved structural patterns in the signaling domains of Toll- and IL-1-like cytokine receptors, and two divergent modular proteins. Figures 2A-2B show a sequence alignment of the common TH domain. TLRs are labeled as in Figure 1; the human (Hu) or mouse (Mo) IL-1 family receptors (IL-1R1-6) are sequentially numbered as earlier proposed (Hardiman, et al., Oncogene 13, 2467 (1996)); Myd88 and the sequences from tobacco (To) and flax, *L. usitatissimum* (Lu), represent C- and N-terminal domains, respectively, of larger, multidomain molecules. Ungapped blocks of sequence (numbered 1-10) are boxed. Triangles indicate deleterious mutations, while truncations N-terminal of the arrow eliminate bioactivity in human IL-1R1 (Heguy, et al., J. Biol. Chem. 267, 2605(1992)). PHD (Rost and Sander, Proteins 19, 55 (1994)) and DSC (King and Sternberg, Protein Sci. 5, 2298 (1996)) secondary structure predictions of α-helix (H), β-strand (E), or coil (L) are marked. The amino

acid shading scheme depicts chemically similar residues: hydrophobic, acidic, basic, Cys, aromatic, structure-breaking, and tiny. Diagnostic sequence patterns for IL-1Rs, TLRs, and full alignment (ALL) were derived by Consensus at a stringency of 75%. Symbols for amino acid subsets are (see internet site for detail): o, alcohol; l, aliphatic; •, any amino acid; a, aromatic; c, charged; h, hydrophobic; -, negative; p, polar; +, positive; s, small; u, tiny; t, turnlike. Figure 2C shows a topology diagram of the proposed TH β/α domain fold. The parallel β-sheet (with β-strands A-E as yellow triangles) is seen at its C-terminal end; α-helices (circles labeled 1-5) link the β-strands; chain connections are to the front (visible) or back (hidden). Conserved, charged residues at the C-end of the β-sheet are noted in gray (Asp) or as a lone black (Arg) residue (see text).

Figure 3 shows evolution of a signaling domain superfamily. The multiple TH module alignment of Figures 2A-2B was used to derive a phylogenetic tree by the Neighbor-Joining method (Thompson, et al., Nucleic Acids Res. 22,4673 (1994)). Proteins labeled as in the alignment; the tree was rendered with TreeView.

Figures 4A-4D depict FISH chromosomal mapping of human TLR genes. Denatured chromosomes from synchronous cultures of human lymphocytes were hybridized to biotinylated TLR cDNA probes for localization. The assignment of the FISH mapping data (left, Figures 4A, TLR2; 4B, TLR3; 4C, TLR4; 4D, TLR5) with chromosomal bands was achieved by superimposing FISH signals with DAPI banded chromosomes (center panels) (Heng and Tsui, Meth. Molec. Biol. 33, 109 (1994)). Analyses are summarized in the form of human chromosome ideograms (right panels).

Figures 5A-5F depict mRNA blot analyses of Human TLRs. Human multiple tissue blots (He, heart; Br, brain; Pl, placenta; Lu, lung; Li, liver; Mu, muscle; Ki, kidney; Pn, Pancreas; Sp, spleen; Th, thymus; Pr, prostate; Te, testis; Ov, ovary, SI, small intestine; Co, colon; PBL, peripheral blood lymphocytes) and cancer cell line (promyelocytic leukemia, HL60; cervical cancer, HELAS3; chronic myelogenous leukemia, K562; lymphoblastic leukemia, Molt4; colorectal adenocarcinoma, SW480; melanoma, G361; Burkitt's Lymphoma Raji, Burkitt's; colorectal adenocarcinoma, SW480; lung carcinoma, A549) containing approximately 2 μg of poly(A)$^+$ RNA per lane were probed with radiolabeled cDNAs encoding TLR1 (Figures 5A-5C), TLR2 (Figure 5D), TLR3 (Figure 5E), and TLR4 (Figure 5F) as described. Blots were exposed

to X-ray film for 2 days (Figures 5A-5C) or one week (Figure 5D-5F) at -70° C with intensifying screens. An anomalous 0.3 kB species appears in some lanes; hybridization experiments exclude a message encoding a TLR cytoplasmic fragment.

SUMMARY OF THE INVENTION

The present invention is directed to nine novel related mammalian receptors, e.g., primate, human, Toll receptor like molecular structures, designated TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, and TLR10, and their biological activities. It includes nucleic acids coding for the polypeptides themselves and methods for their production and use. The nucleic acids of the invention are characterized, in part, by their homology to cloned complementary DNA (cDNA) sequences enclosed herein.

In certain embodiments, the invention provides a composition of matter selected from the group of: a substantially pure or recombinant TLR2 protein or peptide exhibiting identity over a length of at least about 12 amino acids to SEQ ID NO: 4; a natural sequence TLR2 of SEQ ID NO: 4; a fusion protein comprising TLR2 sequence; a substantially pure or recombinant TLR3 protein or peptide exhibiting identity over a length of at least about 12 amino acids to SEQ ID NO: 6; a natural sequence TLR3 of SEQ ID NO: 6; a fusion protein comprising TLR3 sequence; a substantially pure or recombinant TLR4 protein or peptide exhibiting identity over a length of at least about 12 amino acids to SEQ ID NO: 26; a natural sequence TLR4 of SEQ ID NO: 26; a fusion protein comprising TLR4 sequence; a substantially pure or recombinant TLR5 protein or peptide exhibiting identity over a length of at least about 12 amino acids to SEQ ID NO: 10; a natural sequence TLR5 of SEQ ID NO: 10; a fusion protein comprising TLR5 sequence; a substantially pure or recombinant TLR6 protein or peptide exhibiting identity over a length of at least about 12 amino acids to SEQ ID NO: 12, 28, or 30; a natural sequence TLR6 of SEQ ID NO: 12, 28, or 30; a fusion protein comprising TLR6 sequence; a substantially pure or recombinant TLR7 protein or peptide exhibiting identity over a length of at least about 12 amino acids to SEQ ID NO: 16, 18, or 37; a natural sequence TLR7 of SEQ ID NO: 16, 18, or 37; a fusion protein comprising TLR7 sequence; a substantially pure or recombinant TLR8 protein or peptide exhibiting identity over a length of at least about 12 amino acids to SEQ ID NO: 32 or 39; a natural sequence TLR8 of SEQ ID NO: 32 or 39; a fusion protein comprising TLR8

sequence; a substantially pure or recombinant TLR9 protein or peptide exhibiting identity over a length of at least about 12 amino acids to SEQ ID NO: 22 or 41; a natural sequence TLR9 of SEQ ID NO: 22 or 41; a fusion protein comprising TLR9 sequence; a substantially pure or recombinant TLR10 protein or peptide exhibiting identity over a length of at least about 12 amino acids to SEQ ID NO: 34, 43, or 45; a natural sequence TLR10 of SEQ ID NO: 34, 43, or 45; and a fusion protein comprising TLR10 sequence. Preferably, the substantially pure or isolated protein comprises a segment exhibiting sequence identity to a corresponding portion of a TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10, wherein said identity is over at least about 15 amino acids; preferably about 19 amino acids; or more preferably about 25 amino acids. In specific embodiments, the composition of matter: is TLR2, which comprises a mature sequence of SEQ ID NO:4; or lacks a post-translational modification; is TLR3, which comprises a mature sequence of SEQ ID NO:6 ; or lacks a post-translational modification; is TLR4, which: comprises a mature sequence of SEQ ID NO:8 or SEQ ID NO:26; or lacks a post-translational modification; is TLR5, which: comprises the complete sequence of SEQ ID NO:10; or lacks a post-translational; is TLR6, which comprises a mature sequence of SEQ ID NO:12, 14, 28, or 30; or lacks a post-translational modification; is TLR7, which comprises a mature sequence of SEQ ID NO:16, 18, or 37; or lacks a post-translational modification; is TLR8, which: comprises a mature sequence of SEQ ID NO:20, 32, or 39; or lacks a post-translational modification; is TLR9, which: comprises the complete sequence of SEQ ID NO:22 or SEQ ID NO:41; or lacks a post-translational; is TLR10, which comprises a mature sequence of SEQ ID NO:24, 34, 43, or 45; or lacks a post-translational modification; or the composition of matter may be a protein or peptide which: is from a warm blooded animal selected from a mammal, including a primate, such as a human; comprises at least one polypeptide segment of SEQ ID NO: 4, 6, 26, 10, 12, 28, 30, 16, 18, 32, 22, or 34; exhibits a plurality of portions exhibiting said identity; is a natural allelic variant of TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10; has a length at least about 30 amino acids; exhibits at least two non-overlapping epitopes which are specific for a primate TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10; exhibits sequence identity over a length of at least about 35 amino acids to a primate TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9. or TLR10; further exhibits at least two non-overlapping epitopes which are specific for a primate TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10; exhibits identity over a length of at least about 20 amino acids

to a rodent TLR6; is glycosylated; has a molecular weight of at least 100 kD with natural glycosylation; is a synthetic polypeptide; is conjugated to another chemical moiety; is a 5-fold or less substitution from natural sequence; or is a deletion or insertion variant from a natural sequence. In specific embodiments, the TLR, antigenic fragment of TLR, antibody to TLR, antibody fragment to TLR, antibody to a TLR ligand also includes an immobilized form. Immobilization may be by conjugation or attachment to a bead, to a magnetic bead, to a slide, or to a container. Immobilization may be to cyanogen bromide-activated SEPHAROSE by methods well known in the art, or adsorbed to polyolefin surfaces, with or without glutaraldehyde cross-linking.

Other embodiments include a composition comprising: a sterile TLR2 protein or peptide; or the TLR2 protein or peptide and a carrier, wherein the carrier is: an aqueous compound, including water, saline, and/or buffer; and/or formulated for oral, rectal, nasal, topical, or parenteral administration; a sterile TLR3 protein or peptide; or the TLR3 protein or peptide and a carrier, wherein the carrier is: an aqueous compound, including water, saline, and/or buffer; and/or formulated for oral, rectal, nasal, topical, or parenteral administration; a sterile TLR4 protein or peptide; or the TLR4 protein or peptide and a carrier, wherein the carrier is: an aqueous compound, including water, saline, and/or buffer; and/or formulated for oral, rectal, nasal, topical, or parenteral administration; a sterile TLR5 protein or peptide; or the TLR5 protein or peptide and a carrier, wherein the carrier is: an aqueous compound, including water, saline, and/or buffer; and/or formulated for oral, rectal, nasal, topical, or parenteral administration; a sterile TLR6 protein or peptide; or the TLR6 protein or peptide and a carrier, wherein the carrier is: an aqueous compound, including water, saline, and/or buffer; and/or formulated for oral, rectal, nasal, topical, or parenteral administration; a sterile TLR7 protein or peptide; or the TLR7 protein or peptide and a carrier, wherein the carrier is: an aqueous compound, including water, saline, and/or buffer; and/or formulated for oral, rectal, nasal, topical, or parenteral administration; a sterile TLR8 protein or peptide; or the TLR8 protein or peptide and a carrier, wherein the carrier is: an aqueous compound, including water, saline, and/or buffer; and/or formulated for oral, rectal, nasal, topical, or parenteral administration; a sterile TLR9 protein or peptide; or the TLR9 protein or peptide and a carrier, wherein the carrier is: an aqueous compound, including water, saline, and/or buffer; and/or formulated for oral, rectal, nasal, topical, or parenteral administration; a sterile TLR10 protein or peptide; or the

TLR10 protein or peptide and a carrier, wherein the carrier is: an aqueous compound, including water, saline, and/or buffer; and/or formulated for oral, rectal, nasal, topical, or parenteral administration.

In certain fusion protein embodiments, the invention provides a fusion protein comprising: mature protein sequence of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 37, 39, 41, 43, or 45; a detection or purification tag, including a FLAG, His6, or Ig sequence; or sequence of another receptor protein.

Various kit embodiments include a kit comprising a TLR protein or polypeptide, and: a compartment comprising the protein or polypeptide; and/or instructions for use or disposal of reagents in the kit.

Binding compound embodiments include those comprising an antigen binding site from an antibody, which specifically binds to a natural TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10 protein, wherein: the protein is a primate protein; the binding compound is an Fv, Fab, or Fab2 fragment; the binding compound is conjugated to another chemical moiety; or the antibody: is raised against a peptide sequence of a mature polypeptide of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 37, 39, 41, 43, or 45; is raised against a mature TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10; is raised to a purified human TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10; is immunoselected; is a polyclonal antibody; binds to a denatured TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10; exhibits a Kd to antigen of at least 30 μM; is attached to a solid substrate, including a bead or plastic membrane; is in a sterile composition; or is detectably labeled, including a radioactive or fluorescent label. A binding composition kit often comprises the binding compound, and: a compartment comprising said binding compound; and/or instructions for use or disposal of reagents in the kit. Often the kit is capable of making a qualitative or quantitative analysis.

Methods are provided, e.g., of making an antibody, comprising immunizing an immune system with an immunogenic amount of a primate TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10, thereby causing said antibody to be produced; or producing an antigen:antibody complex, comprising contacting such an antibody with a mammalian TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10 protein or peptide, thereby allowing said complex to form.

Other compositions include a composition comprising: a sterile binding compound, or the binding compound and a carrier, wherein the carrier is: an aqueous compound, including water, saline, and/or buffer; and/or formulated for oral, rectal, nasal, topical, or parenteral administration.

Nucleic acid embodiments include an isolated or recombinant nucleic acid encoding a TLR2-10 protein or peptide or fusion protein, wherein: the TLR is from a mammal; or the nucleic acid: encodes an antigenic peptide sequence of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 37, 39, 41, 43, or 45; encodes a plurality of antigenic peptide sequences of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 37, 39, 41, 43, or 45; comprises at least 17 contiguous nucleotides from SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 36, 38, 40, 42, or 44; exhibits at least about 80% identity to a natural cDNA encoding said segment; is an expression vector; further comprises an origin of replication; is from a natural source; comprises a detectable label such as a radioactive label, a fluorescent label, or an immunogenic label; comprises synthetic nucleotide sequence; is less than 6 kb, preferably less than 3 kb; is from a mammal, including a primate; comprises a natural full length coding sequence; is a hybridization probe for a gene encoding said TLR; or is a PCR primer, PCR product, or mutagenesis primer. A cell, tissue, or organ comprising such a recombinant nucleic acid is also provided. Preferably, the cell is: a prokaryotic cell; a eukaryotic cell; a bacterial cell; a yeast cell; an insect cell; a mammalian cell; a mouse cell; a primate cell; or a human cell. Kits are provided comprising such nucleic acids, and: a compartment comprising said nucleic acid; a compartment further comprising a primate TLR2, TLR3, TLR4, or TLR5 protein or polypeptide; and/or instructions for use or disposal of reagents in the kit. Often, the kit is capable of making a qualitative or quantitative analysis.

Other embodiments include a nucleic acid which: hybridizes under wash conditions of 30° C and less than 2M salt to SEQ ID NO: 3; hybridizes under wash conditions of 30° C and less than 2 M salt to SEQ ID NO: 5; hybridizes under wash conditions of 30° C and less than 2M salt to SEQ ID NO: 7; hybridizes under wash conditions of 30° C and less than 2 M salt to SEQ ID NO: 9; hybridizes under wash conditions of 30° C and less than 2 M salt to SEQ ID NO: 11, 13, 27, or 29; hybridizes under wash conditions of 30° C and less than 2 M salt to SEQ ID NO: 15, 17, or 36; hybridizes under wash conditions of 30° C and less than 2 M salt to SEQ ID NO: 19, 31, or 38; hybridizes under wash conditions of 30° C and less than 2 M salt to SEQ ID NO:

21 or 40; hybridizes under wash conditions of 30° C and less than 2 M salt to SEQ ID NO: 23, 33, 42, or 44; exhibits at least about 85% identity over a stretch of at least about 30 nucleotides to a primate TLR2; exhibits at least about 85% identity over a stretch of at least about 30 nucleotides to a primate TLR3; exhibits at least about 85% identity over a stretch of at least about 30 nucleotides to a primate TLR4; or exhibits at least about 85% identity over a stretch of at least about 30 nucleotides to a primate TLR5. Preferably, such nucleic acid will have such properties, wherein: wash conditions are at 45° C and/or 500 mM salt; or the identity is at least 90% and/or the stretch is at least 55 nucleotides.

More preferably, the wash conditions are at 55° C and/or 150 mM salt; or the identity is at least 95% and/or the stretch is at least 75 nucleotides.

Also provided are methods of producing a ligand:receptor complex, comprising contacting a substantially pure primate TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10, including a recombinant or synthetically produced protein, with candidate Toll ligand; thereby allowing said complex to form.

The invention also provides a method of modulating physiology or development of a cell or tissue culture cells comprising contacting the cell with an agonist or antagonist of a mammalian TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10. Preferably, the cell is a pDC2 cell with the agonist or antagonist of TLR10.

Abbreviations: TLR, Toll-like receptor; DTLR, DNAX Toll-like receptor; IL-1R, interleukin-1 receptor; TH, Toll homology; LRR, leucine-rich repeat; EST, expressed sequence tag; STS, sequence tagged site; FISH, fluorescence in situ hybridization; GMCSF, granulocyte-macrophage colony-stimulating factor; NIPC or IPC, natural interferon producing cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

OUTLINE

I. General

II. Activities

III. Nucleic acids

- A. encoding fragments, sequence, probes
- B. mutations, chimeras, fusions
- C. making nucleic acids
- D. vectors, cells comprising

IV. Proteins, Peptides

- A. fragments, sequence, immunogens, antigens
- B. muteins
- C. agonists/antagonists, functional equivalents
- D. making proteins
- E. soluble receptors

V. Making nucleic acids, proteins

- A. synthetic
- B. recombinant
- C. natural sources

VI. Antibodies

- A. polyclonals
- B. monoclonal
- C. fragments; Kd
- D. anti-idiotypic antibodies
- E. hybridoma cell lines

VII. Kits and Methods to quantify TLRs 2-10

- A. ELISA
- B. assay mRNA encoding
- C. qualitative/quantitative
- D. kits

VIII. Therapeutic compositions, methods

A. combination compositions

B. unit dose

C. administration

IX. Ligands

I. General

The present invention provides the amino acid sequence and DNA sequence of mammalian, herein primate Toll like receptor molecules (TLR) having particular defined properties, both structural and biological. These have been designated herein as TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10, respectively, and increase the number of members of the human Toll like receptor family from 1 to 10. Various cDNAs encoding these molecules were obtained from primate, e.g., human, cDNA sequence libraries. Other primate or other mammalian counterparts would also be desired.

Some of the standard methods applicable are described or referenced, e.g., in Maniatis, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press (1982); Sambrook, et al., Molecular Cloning: A Laboratory Manual, (2d ed.), vols. 1-3, CSH Press, NY (1989); and Ausubel, et al., Current Protocols in Molecular Biology, Greene/Wiley, New York (1987); each of which is incorporated herein by reference.

A complete nucleotide (SEQ ID NO: 1) and corresponding amino acid sequence (SEQ. ID NO: 2) of a human TLR1 coding segment is shown in the indicated sequence listings. See also Nomura, et al., DNA Res. 1,27 (1994). A complete nucleotide (SEQ ID NO: 3) and corresponding amino acid sequence (SEQ ID NO: 4) of a human TLR2 coding segment is also shown, as indicated. A complete nucleotide (SEQ ID NO: 5) and corresponding amino acid sequence (SEQ ID NO: 6) of a human TLR3 coding segment are shown, as indicated. A complete nucleotide (SEQ ID NO: 7) and corresponding amino acid sequence (SEQ ID NO: 8) of a human TLR4 coding segment are also shown, in the indicated sequence listings. See also SEQ ID NO: 25 and 26. A partial nucleotide (SEQ ID NO: 9) and corresponding amino acid sequence (SEQ ID NO: 10) of a human TLR5 coding segment are shown in the indicated sequence listings. A complete nucleotide (SEQ ID NO: 11) and corresponding amino acid sequence (SEQ ID NO: 12) of a human TLR6 coding segment are shown, along with partial

sequence of a mouse TLR6 (SEQ ID NO: 13, 14, 27, 28, 29, and 30), as indicated. Partial nucleotide (SEQ ID NO: 15 and 17) and corresponding amino acid sequence (SEQ ID NO: 16 and 18) of a human TLR7 coding segment are shown in the indicated sequence listings, while full length sequences are provided in SEQ ID NO: 36 and 37. Partial nucleotide (SEQ ID NO: 19) and corresponding amino acid sequence (SEQ ID NO: 20) of a human TLR8 coding segment is shown, with supplementary sequence (SEQ ID NO: 31, 32, 38, and 39). Partial nucleotide (SEQ ID NO: 21) and corresponding amino acid sequence (SEQ ID NO: 22) of a human TLR9 coding segment is shown in the indicated sequence listings. See also SEQ ID NO: 40 and 41. Partial nucleotide (SEQ ID NO: 23) and corresponding amino acid sequence (SEQ ID NO: 24) of a human TLR10 coding segment is shown as indicated, along with supplementary sequences (SEQ ID NO: 33, 34, 42, and 43) and rodent, e.g., mouse, sequence (SEQ ID NO: 35, 44, and 45).

Transmembrane segments correspond approximately to 802-818 (791-823) of primate TLR7 SEQ ID NO: 37; 559-575 (550-586) of TLR8 SEQ ID NO: 39; 553-569 (549-582) of TLR9 SEQ ID NO: 41; 796-810 (790-814) of TLR10 SEQ ID NO: 43; and 481-497 (475-503) of TLR10 SEQ ID NO: 45.

As used herein, the term Toll like receptor 2 (TLR2) shall be used to describe a protein comprising a protein or peptide segment having or sharing the amino acid sequence shown in SEQ ID NO: 4, or a substantial fragment thereof. Similarly, with a TLR3 and SEQ ID NO: 6; TLR4 and SEQ ID NO: 8; TLR5 and SEQ ID NO: 9; TLR6 and SEQ ID NO: 12; TLR7 and SEQ ID NO: 37; TLR8 and SEQ ID NO: 20; TLR9 and SEQ ID NO: 22; and TLR10 and SEQ ID NO: 24. Rodent, e.g., mouse, TLR11 sequence is provided, e.g., in EST AA739083; TLR13 in ESTAI019567; TLR14 in ESTs AI390330 and AA244663.

The invention also includes a protein variations of the respective TLR allele whose sequence is provided, e.g., a mutein agonist or antagonist. Typically, such agonists or antagonists will exhibit less than about 10% sequence differences, and thus will often have between 1- and 11-fold substitutions, e.g., 2-, 3-, 5-, 7-fold, and others. It also encompasses allelic and other variants, e.g., natural polymorphic, of the protein described. Typically, it will bind to its corresponding biological receptor with high affinity, e.g., at least about 100 nM, usually better than about 30 nM, preferably better than about 10 nM, and more preferably at

better than about 3 nM. The term shall also be used herein to refer to related naturally occurring forms, e.g., alleles, polymorphic variants, and metabolic variants of the mammalian protein.

This invention also encompasses proteins or peptides having substantial amino acid sequence identity with the amino acid sequence in Table 2. It will include sequence variants with relatively few substitutions, e.g., preferably less than about 3-5. Similar features apply to the other TLR sequences provided in Tables 3, 4, 5, 6, 7, 8, 9, or 10.

A substantial polypeptide "fragment", or "segment", is a stretch of amino acid residues of at least about 8 amino acids, generally at least 10 amino acids, more generally at least 12 amino acids, often at least 14 amino acids, more often at least 16 amino acids, typically at least 18 amino acids, more typically at least 20 amino acids, usually at least 22 amino acids, more usually at least 24 amino acids, preferably at least 26 amino acids, more preferably at least 28 amino acids, and, in particularly preferred embodiments, at least about 30 or more amino acids. Sequences of segments of different proteins can be compared to one another over appropriate length stretches.

Amino acid sequence homology, or sequence identity, is determined by optimizing residue matches, if necessary, by introducing gaps as required. See, e.g., Needleham, et al., J. Mol. Biol. 48,443 (1970); Sankoff, et al., Chapter One in Time Warps, String Edits, and Macromolecules: The Theory and Practice of Sequence Comparison, Addison-Wesley, Reading, MA (1983); and software packages from IntelliGenetics, Mountain View, CA; GCG WISCONSIN PACKAGE (Accelrys, Inc., San Diego, CA); and the NCBI (NIH); each of which is incorporated herein by reference. This changes when considering conservative substitutions as matches. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Homologous amino acid sequences are intended to include natural allelic and interspecies variations in the cytokine sequence. Typical homologous proteins or peptides will have from 50-100% homology (if gaps can be introduced), to 60-100% homology (if conservative substitutions are included) with an amino acid sequence segment of SEQ ID NO. 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 37, 39, 41, 43, or 45. Homology measures will be at least about 70%, generally at least 76%, more generally at least 81%, often at least 85%, more often at least 88%, typically at least 90%, more typically at least 92%, usually at least 94%, more usually at least 95%, preferably at

least 96%, and more preferably at least 97%, and in particularly preferred embodiments, at least 98% or more. The degree of homology will vary with the length of the compared segments. Homologous proteins or peptides, such as the allelic variants, will share most biological activities with the embodiments described in SEQ ID NO. 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 37, 39, 41, 43, or 45.. Particularly interesting regions of comparison, at the amino acid or nucleotide levels, correspond to those within each of the blocks 1-10, or intrablock regions, corresponding to those indicated in Figures 2A-2B.

As used herein, the term "biological activity" is used to describe, without limitation, effects on inflammatory responses, innate immunity, and/or morphogenic development by respective ligands. For example, these receptors should, like IL-1 receptors, mediate phosphatase or phosphorylase activities, which activities are easily measured by standard procedures. See, e.g., Hardie, et al., The Protein Kinase FactBook vols. I and II, Academic Press, San Diego, CA (1995); Hanks, et al., Meth. Enzymol. 200, 38(1991); Hunter, et al., Cell 70, 375 (1992); Lewin, Cell 61, 743 (1990); Pines, et al., Cold Spring Harbor Symp. Quant. Biol. 56, 449 (1991); and Parker, et al., Nature 363, 736 (1993). The receptors exhibit biological activities much like regulatable enzymes, regulated by ligand binding. However, the enzyme turnover number is more close to an enzyme than a receptor complex. Moreover, the numbers of occupied receptors necessary to induce such enzymatic activity is less than most receptor systems, and may number closer to dozens per cell, in contrast to most receptors which will trigger at numbers in the thousands per cell. The receptors, or portions thereof, may be useful as phosphate labeling enzymes to label general or specific substrates.

The terms ligand, agonist, antagonist, and analog of, e.g., a TLR, include molecules that modulate the characteristic cellular responses to Toll ligand like proteins, as well as molecules possessing the more standard structural binding competition features of ligand-receptor interactions, e.g., where the receptor is a natural receptor or an antibody. The cellular responses likely are mediated through binding of various Toll ligands to cellular receptors related to, but possibly distinct from, the type I or type II IL-1 receptors. See, e.g., Belvin and Anderson, Ann. Rev. Cell Dev. Biol. 12, 393 (1996); Morisato and Anderson, Ann. Rev. Genetics 29, 371 (1995) and Hultmark, Nature 367, 116 (1994).

Also, a ligand is a molecule which serves either as a natural ligand to which said receptor, or an analog thereof, binds, or a molecule which is a functional analog of the natural ligand. The

functional analog may be a ligand with structural modifications, or may be a wholly unrelated molecule which has a molecular shape which interacts with the appropriate ligand binding determinants. The ligands may serve as agonists or antagonists, see, e.g., Goodman, et al., Goodman & Gilman's: The Pharmacological Bases of Therapeutics, Pergamon Press, New York (1990).

Rational drug design may also be based upon structural studies of the molecular shapes of a receptor or antibody and other effectors or ligands. Effectors may be other proteins which mediate other functions in response to ligand binding, or other proteins which normally interact with the receptor. One means for determining which sites interact with specific other proteins is a physical structure determination, e.g., x-ray crystallography or 2 dimensional NMR techniques. These will provide guidance as to which amino acid residues form molecular contact regions. For a detailed description of protein structural determination, see, e.g., Blundell and Johnson, Protein Crystallography, Academic Press, New York (1976), which is hereby incorporated herein by reference.

II. Activities

The Toll like receptor proteins will have a number of different biological activities, e.g., in phosphate metabolism, being added to or removed from specific substrates, typically proteins. Such will generally result in modulation of an inflammatory function, other innate immunity response, or a morphological effect. The TLR2, 3, 4, 5, 6, 7, 8, 9, or 10 proteins are homologous to other Toll like receptor proteins, but each have structural differences. For example, a human TLR2 gene coding sequence probably has about 70% identity with the nucleotide coding sequence of mouse TLR2. At the amino acid level, there is also likely to be reasonable identity.

The biological activities of the TLRs will be related to addition or removal of phosphate moieties to substrates, typically in a specific manner, but occasionally in a non specific manner. Substrates may be identified, or conditions for enzymatic activity may be assayed by standard methods, e.g., as described in Hardie, et al., The Protein Kinase FactBook vols. I and II, Academic Press, San Diego, CA (1995); Hanks, et al., Meth. Enzymol. 200, 38 (1991); Hunter, et al., Cell 70, 375 (1992); Lewin, Cell 61, 743 (1990); Pines, et al., Cold Spring Harbor Symp. Quant. Biol. 56, 449 (1991); and Parker, et al., Nature 363, 736 (1993).

III. Nucleic Acids

This invention contemplates use of isolated nucleic acid or fragments, e.g., which encode these or closely related proteins, or fragments thereof, e.g., to encode a corresponding polypeptide, preferably one which is biologically active. In addition, this invention covers isolated or recombinant DNA which encodes such proteins or polypeptides having characteristic sequences of the respective TLRs, individually or as a group. Typically, the nucleic acid is capable of hybridizing, under appropriate conditions, with a nucleic acid sequence segment shown in SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 36, 38, 40, 42, or 44, but preferably not with a corresponding segment of SEQ ID NO:1. Said biologically active protein or polypeptide can be a full length protein, or fragment, and will typically have a segment of amino acid sequence highly homologous to one shown in SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 37, 39, 41, 43, or 45. Further, this invention covers the use of isolated or recombinant nucleic acid, or fragments thereof, which encode proteins having fragments which are equivalent to the TLR2-10 proteins. The isolated nucleic acids can have the respective regulatory sequences in the 5' and 3' flanks, e.g., promoters, enhancers, poly-A addition signals, and others from the natural gene.

An "isolated" nucleic acid is a nucleic acid, e.g., an RNA, DNA, or a mixed polymer, which is substantially pure, e.g., separated from other components which naturally accompany a native sequence, such as ribosomes, polymerases, and flanking genomic sequences from the originating species. The term embraces a nucleic acid sequence which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates, which are thereby distinguishable from naturally occurring compositions, and chemically synthesized analogs or analogs biologically synthesized by heterologous systems. A substantially pure molecule includes isolated forms of the molecule, either completely or substantially pure.

An isolated nucleic acid will generally be a homogeneous composition of molecules, but will, in some embodiments, contain heterogeneity, preferably minor. This heterogeneity is typically found at the polymer ends or portions not critical to a desired biological function or activity.

A "recombinant" nucleic acid is typically defined either by its method of production or its structure. In reference to its method of production, e.g., a product made by a process, the process is use of recombinant nucleic acid techniques, e.g., involving human intervention in the

nucleotide sequence. Typically this intervention involves in vitro manipulation, although under certain circumstances it may involve more classical animal breeding techniques. Alternatively, it can be a nucleic acid made by generating a sequence comprising fusion of two fragments which are not naturally contiguous to each other, but is meant to exclude products of nature, e.g., naturally occurring mutants as found in their natural state. Thus, for example, products made by transforming cells with any unnaturally occurring vector is encompassed, as are nucleic acids comprising sequence derived using any synthetic oligonucleotide process. Such a process is often done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a restriction enzyme sequence recognition site. Alternatively, the process is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the commonly available natural forms, e.g., encoding a fusion protein. Restriction enzyme recognition sites are often the target of such artificial manipulations, but other site specific targets, e.g., promoters, DNA replication sites, regulation sequences, control sequences, or other useful features may be incorporated by design. A similar concept is intended for a recombinant, e.g., fusion, polypeptide. This will include a dimeric repeat. Specifically included are synthetic nucleic acids which, by genetic code redundancy, encode equivalent polypeptides to fragments of TLR2-5 and fusions of sequences from various different related molecules, e.g., other IL-1 receptor family members.

A "fragment" in a nucleic acid context is a contiguous segment of at least about 17 nucleotides, generally at least 21 nucleotides, more generally at least 25 nucleotides, ordinarily at least 30 nucleotides, more ordinarily at least 35 nucleotides, often at least 39 nucleotides, more often at least 45 nucleotides, typically at least 50 nucleotides, more typically at least 55 nucleotides, usually at least 60 nucleotides, more usually at least 66 nucleotides, preferably at least 72 nucleotides, more preferably at least 79 nucleotides, and in particularly preferred embodiments will be at least 85 or more nucleotides. Typically, fragments of different genetic sequences can be compared to one another over appropriate length stretches, particularly defined segments such as the domains described below.

A nucleic acid which codes for a TLR2-10 will be particularly useful to identify genes, mRNA, and cDNA species which code for itself or closely related proteins, as well as DNAs which code for polymorphic, allelic, or other genetic variants, e.g., from different individuals or

related species. Preferred probes for such screens are those regions of the interleukin which are conserved between different polymorphic variants or which contain nucleotides which lack specificity, and will preferably be full length or nearly so. In other situations, polymorphic variant specific sequences will be more useful.

This invention further covers recombinant nucleic acid molecules and fragments having a nucleic acid sequence identical to or highly homologous to the isolated DNA set forth herein. In particular, the sequences will often be operably linked to DNA segments which control transcription, translation, and DNA replication. These additional segments typically assist in expression of the desired nucleic acid segment.

Homologous, or highly identical, nucleic acid sequences, when compared to one another or Table 2-10 sequences, exhibit significant similarity. The standards for homology in nucleic acids are either measures for homology generally used in the art by sequence comparison or based upon hybridization conditions. Comparative hybridization conditions are described in greater detail below.

Substantial identity in the nucleic acid sequence comparison context means either that the segments, or their complementary strands, when compared, are identical when optimally aligned, with appropriate nucleotide insertions or deletions, in at least about 60% of the nucleotides, generally at least 66%, ordinarily at least 71%, often at least 76%, more often at least 80%, usually at least 84%, more usually at least 88%, typically at least 91%, more typically at least about 93%, preferably at least about 95%, more preferably at least about 96 to 98% or more, and in particular embodiments, as high at about 99% or more of the nucleotides, including, e.g., segments encoding structural domains such as the segments described below. Alternatively, substantial identity will exist when the segments will hybridize under selective hybridization conditions, to a strand or its complement, typically using a sequence derived from Tables 2-10. Typically, selective hybridization will occur when there is at least about 55% homology over a stretch of at least about 14 nucleotides, more typically at least about 65%, preferably at least about 75%, and more preferably at least about 90%. See, Kanehisa, Nucl. Acids Res. 12, 203 (1984), which is incorporated herein by reference. The length of homology comparison, as described, may be over longer stretches, and in certain embodiments will be over a stretch of at least about 17 nucleotides, generally at least about 20 nucleotides, ordinarily at least about 24 nucleotides, usually at least about 28 nucleotides, typically at least about 32 nucleotides, more

typically at least about 40 nucleotides, preferably at least about 50 nucleotides, and more preferably at least about 75 to 100 or more nucleotides.

Stringent conditions, in referring to homology in the hybridization context, will be stringent combined conditions of salt, temperature, organic solvents, and other parameters typically controlled in hybridization reactions. Stringent temperature conditions will usually include temperatures in excess of about 30° C, more usually in excess of about 37° C, typically in excess of about 45° C, more typically in excess of about 55° C, preferably in excess of about 65° C, and more preferably in excess of about 70° C. Stringent salt conditions will ordinarily be less than about 500 mM, usually less than about 400 mM, more usually less than about 300 mM, typically less than about 200 mM, preferably less than about 100 mM, and more preferably less than about 80 mM, even down to less than about 20 mM. However, the combination of parameters is much more important than the measure of any single parameter. See, e.g., Wetmur and Davidson, J. Mol. Biol. 31, 349 (1968), which is hereby incorporated herein by reference.

Alternatively, for sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optical alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and Waterman, Adv. Appl. Math. 2, 482 (1981), by the homology alignment algorithm of Needlman and Wunsch, J. Mol. Biol. 48, 443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Nat'l Acad. Sci. USA 85, 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see generally Ausubel et al., supra).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle, J. Mol. Evol. 35, 351 (1987). The method

used is similar to the method described by Higgins and Sharp, CABIOS 5, 151 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described Altschul, et al., J. Mol. Biol. 215, 403 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http:www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul, et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff, Proc. Nat'l Acad. Sci. USA 89, 10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul, Proc. Nat'l Acad. Sci. USA 90, 5873 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

A further indication that two nucleic acid sequences of polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, e.g., where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described below.

The isolated DNA can be readily modified by nucleotide substitutions, nucleotide deletions, nucleotide insertions, and inversions of nucleotide stretches. These modifications result in novel DNA sequences which encode this protein or its derivatives. These modified sequences can be used to produce mutant proteins (muteins) or to enhance the expression of variant species. Enhanced expression may involve gene amplification, increased transcription, increased translation, and other mechanisms. Such mutant TLR–like derivatives include predetermined or site-specific mutations of the protein or its fragments, including silent mutations using genetic code degeneracy. "Mutant TLR" as used herein encompasses a polypeptide otherwise falling within the homology definition of the TLR as set forth above, but having an amino acid sequence which differs from that of other TLR-like proteins as found in nature, whether by way of deletion, substitution, or insertion. In particular, "site specific mutant TLR" encompasses a protein having substantial homology with a protein of Tables 2-10, and typically shares most of the biological activities or effects of the forms disclosed herein.

Although site specific mutation sites are predetermined, mutants need not be site specific. Mammalian TLR mutagenesis can be achieved by making amino acid insertions or deletions in the gene, coupled with expression. Substitutions, deletions, insertions, or any combinations may

be generated to arrive at a final construct. Insertions include amino- or carboxy- terminal fusions. Random mutagenesis can be conducted at a target codon and the expressed mammalian TLR mutants can then be screened for the desired activity. Methods for making substitution mutations at predetermined sites in DNA having a known sequence are well known in the art, e.g., by M13 primer mutagenesis. See also Sambrook, et al. (1989) and Ausubel, et al. (1987 and periodic Supplements).

The mutations in the DNA normally should not place coding sequences out of reading frames and preferably will not create complementary regions that could hybridize to produce secondary mRNA structure such as loops or hairpins.

The phosphoramidite method described by Beaucage and Carruthers, Tetra. Letts. 22, 1859 (1981), will produce suitable synthetic DNA fragments. A double stranded fragment will often be obtained either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Polymerase chain reaction (PCR) techniques can often be applied in mutagenesis. Alternatively, mutagenesis primers are commonly used methods for generating defined mutations at predetermined sites. See, e.g., Innis, et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, CA (1990); and Dieffenbach and Dveksler, PCR Primer: A Laboratory Manual, Cold Spring Harbor Press, CSH, NY (1995).

IV. Proteins and Peptides

As described above, the present invention encompasses primate TLR2-10, e.g., whose sequences are disclosed in SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 37, 39, 41, 43, or 45., and described above. Allelic and other variants are also contemplated, including, e.g., fusion proteins combining portions of such sequences with others, including epitope tags and functional domains.

The present invention also provides recombinant proteins, e.g., heterologous fusion proteins using segments from these rodent proteins. A heterologous fusion protein is a fusion of proteins or segments which are naturally not normally fused in the same manner. Thus, the fusion product of a TLR with an IL-1 receptor is a continuous protein molecule having sequences fused in a typical peptide linkage, typically made as a single translation product and

exhibiting properties, e.g., sequence or antigenicity, derived from each source peptide. A similar concept applies to heterologous nucleic acid sequences.

In addition, new constructs may be made from combining similar functional or structural domains from other related proteins, e.g., IL-1 receptors or other TLRs, including species variants. For example, ligand-binding or other segments may be "swapped" between different new fusion polypeptides or fragments. See, e.g., Cunningham, et al., Science 243, 1330 (1989); and O'Dowd, et al., J. Biol. Chem. 263,15985 (1988), each of which is incorporated herein by reference. Thus, new chimeric polypeptides exhibiting new combinations of specificities will result from the functional linkage of receptor-binding specificities. For example, the ligand binding domains from other related receptor molecules may be added or substituted for other domains of this or related proteins. The resulting protein will often have hybrid function and properties. For example, a fusion protein may include a targeting domain which may serve to provide sequestering of the fusion protein to a particular subcellular organelle.

Candidate fusion partners and sequences can be selected from various sequence data bases, e.g., GenBank, c/o IntelliGenetics, Mountain View, CA; and BCG, University of Wisconsin Biotechnology Computing Group, Madison, WI, which are each incorporated herein by reference.

The present invention particularly provides muteins which bind Toll ligands, and/or which are affected in signal transduction. Structural alignment of human TLR1-10 with other members of the IL-1 family show conserved features/residues. See, e.g., Figure 3A. Alignment of the human TLR sequences with other members of the IL-1 family indicates various structural and functionally shared features. See also, Bazan, et al., Nature 379, 591 (1996); Lodi, et al., Science 263, 1762 (1994); Sayle and Milner-White, TIBS 20, 374 (1995); and Gronenberg, et al., Protein Engineering 4, 263 (1991).

The IL-1α and IL-1β ligands bind an IL-1 receptor type I as the primary receptor and this complex then forms a high affinity receptor complex with the IL-1 receptor type III. Such receptor subunits are probably shared with the new IL-1 family members.

Similar variations in other species counterparts of TLR2-10 sequences, e.g., in the corresponding regions, should provide similar interactions with ligand or substrate. Substitutions with either mouse sequences or human sequences are particularly preferred. Conversely,

conservative substitutions away from the ligand binding interaction regions will probably preserve most signaling activities.

"Derivatives" of the primate TLR2-10 include amino acid sequence mutants, glycosylation variants, metabolic derivatives and covalent or aggregative conjugates with other chemical moieties. Covalent derivatives can be prepared by linkage of functionalities to groups which are found in the TLR amino acid side chains or at the N- or C- termini, e.g., by means which are well known in the art. These derivatives can include, without limitation, aliphatic esters or amides of the carboxyl terminus, or of residues containing carboxyl side chains, O-acyl derivatives of hydroxyl group-containing residues, and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g., lysine or arginine. Acyl groups are selected from the group of alkyl-moieties including C3 to C18 normal alkyl, thereby forming alkanoyl aroyl species.

In particular, glycosylation alterations are included, e.g., made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing, or in further processing steps. Particularly preferred means for accomplishing this are by exposing the polypeptide to glycosylating enzymes derived from cells which normally provide such processing, e.g., mammalian glycosylation enzymes. Deglycosylation enzymes are also contemplated. Also embraced are versions of the same primary amino acid sequence which have other minor modifications, including phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

A major group of derivatives are covalent conjugates of the receptors or fragments thereof with other proteins of polypeptides. These derivatives can be synthesized in recombinant culture such as N- or C-terminal fusions or by the use of agents known in the art for their usefulness in cross-linking proteins through reactive side groups. Preferred derivatization sites with cross-linking agents are at free amino groups, carbohydrate moieties, and cysteine residues.

Fusion polypeptides between the receptors and other homologous or heterologous proteins are also provided. Homologous polypeptides may be fusions between different receptors, resulting in, for instance, a hybrid protein exhibiting binding specificity for multiple different Toll ligands, or a receptor which may have broadened or weakened specificity of substrate effect. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. Typical examples are fusions

of a reporter polypeptide, e.g., luciferase, with a segment or domain of a receptor, e.g., a ligand-binding segment, so that the presence or location of a desired ligand may be easily determined. See, e.g., Dull, et al., U.S. Patent No. 4,859,609, which is hereby incorporated herein by reference. Other gene fusion partners include glutathione-S-transferase (GST), bacterial ß-galactosidase, trpE, Protein A, ß-lactamase, alpha amylase, alcohol dehydrogenase, and yeast alpha mating factor. See, e.g., Godowski, et al., Science 241, 812 (1988).

Such polypeptides may also have amino acid residues which have been chemically modified by phosphorylation, sulfonation, biotinylation, or the addition or removal of other moieties, particularly those which have molecular shapes similar to phosphate groups. In some embodiments, the modifications will be useful labeling reagents, or serve as purification targets, e.g., affinity ligands.

Fusion proteins will typically be made by either recombinant nucleic acid methods or by synthetic polypeptide methods. Techniques for nucleic acid manipulation and expression are described generally, for example, in Sambrook, et al., Molecular Cloning: A Laboratory Manual (2d ed.), Vols. 1-3, Cold Spring Harbor Laboratory (1989), and Ausubel, et al., Current Protocols in Molecular Biology, Greene/Wiley, New York (1987), which are each incorporated herein by reference. Techniques for synthesis of polypeptides are described, for example, in Merrifield, J. Amer. Chem. Soc. 85, 2149 (1963); Merrifield, Science 232, 341 (1986); and Atherton, et al., Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford (1989); each of which is incorporated herein by reference. See also Dawson, et al., Science 266,776 (1994)for methods to make larger polypeptides.

This invention also contemplates the use of derivatives of a TLR2-10 other than variations in amino acid sequence or glycosylation. Such derivatives may involve covalent or aggregative association with chemical moieties. These derivatives generally fall into three classes: (1) salts, (2) side chain and terminal residue covalent modifications, and (3) adsorption complexes, for example with cell membranes. Such covalent or aggregative derivatives are useful as immunogens, as reagents in immunoassays, or in purification methods such as for affinity purification of a receptor or other binding molecule, e.g., an antibody. For example, a Toll ligand can be immobilized by covalent bonding to a solid support such as cyanogen bromide-activated Sepharose, by methods which are well known in the art, or adsorbed onto polyolefin surfaces, with or without glutaraldehyde cross-linking, for use in the assay or

purification of a TLR receptor, antibodies, or other similar molecules. The ligand can also be labeled with a detectable group, for example radioiodinated by the chloramine T procedure, covalently bound to rare earth chelates, or conjugated to another fluorescent moiety for use in diagnostic assays.

Soluble Toll-like receptors (sTLR) as used in the context of the present invention refers to a protein, or a substantially equivalent analog, having an amino acid sequence corresponding to the extracellular region of native TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10. Soluble TLRs may be constructed by deleting terminal or internal residues or sequences. Particularly preferred sequences include those in which the transmembrane region and intracellular domain of a TLR are deleted or substituted with hydrophilic residues to facilitate secretion of the receptor into the cell culture medium. Software programs can be used for predicting the transmembrane, extracellular, and cytosolic domains of a polypeptide. These software programs can be found in the GCG WISCONSIN PACKAGE (Accelrys, Inc., San Diego, CA) and in the LASERGENE sequence analysis software (DNAStar, Inc., Madison, WI). The resulting water-soluble protein is referred to as a soluble TLR molecule, where this TLR retains its ability to bind its ligand, e.g., bacterial lipopolysaccharide, endotoxin, peptidoglycan, lipoteichoic acid, and unmethylated CpG oligonucleotides.

When administered in therapeutic formulations, soluble TLRs circulate in the body and bind to its ligand or ligands, where the ligands may be soluble, intracellular, intercellular, or occurring as part of a microbe or fungus. When the soluble TLR binds to the ligand, the ligand is prevented from interacting with its natural TLR, and thereby prevented from relaying a signal to the cell.

DNA constructs coding for soluble TLRs can be inserted in appropriate expression vectors, expressed in cultured cells or microorganisms, and expressed. The expressed soluble TLR can be assayed for the ability to bind the above mentioned ligands (See, e.g., U.S. Patent No. 5,767,065, issued to Mosley, et al.; U.S. Patent No. 5,712,155, issued to Smith, et al.)

V. Making Nucleic Acids and Protein

DNA which encodes the protein or fragments thereof can be obtained by chemical synthesis, screening cDNA libraries, or by screening genomic libraries prepared from a wide

variety of cell lines or tissue samples. Natural sequences can be isolated using standard methods and the sequences provided herein, e.g., in Tables 2-10. Other species counterparts can be identified by hybridization techniques, or by various PCR techniques, combined with or by searching in sequence databases, e.g., GenBank.

This DNA can be expressed in a wide variety of host cells for the synthesis of a full-length receptor or fragments which can in turn, for example, be used to generate polyclonal or monoclonal antibodies; for binding studies; for construction and expression of modified ligand binding or kinase/phosphatase domains; and for structure/function studies. Variants or fragments can be expressed in host cells that are transformed or transfected with appropriate expression vectors. These molecules can be substantially free of protein or cellular contaminants, other than those derived from the recombinant host, and therefore are particularly useful in pharmaceutical compositions when combined with a pharmaceutically acceptable carrier and/or diluent. The protein, or portions thereof, may be expressed as fusions with other proteins.

Expression vectors are typically self-replicating DNA or RNA constructs containing the desired receptor gene or its fragments, usually operably linked to suitable genetic control elements that are recognized in a suitable host cell. These control elements are capable of effecting expression within a suitable host. The specific type of control elements necessary to effect expression will depend upon the eventual host cell used. Generally, the genetic control elements can include a prokaryotic promoter system or a eukaryotic promoter expression control system, and typically include a transcriptional promoter, an optional operator to control the onset of transcription, transcription enhancers to elevate the level of mRNA expression, a sequence that encodes a suitable ribosome binding site, and sequences that terminate transcription and translation. Expression vectors also usually contain an origin of replication that allows the vector to replicate independently of the host cell.

The vectors of this invention include those which contain DNA which encodes a protein, as described, or a fragment thereof encoding a biologically active equivalent polypeptide. The DNA can be under the control of a viral promoter and can encode a selection marker. This invention further contemplates use of such expression vectors which are capable of expressing eukaryotic cDNA coding for such a protein in a prokaryotic or eukaryotic host, where the vector is compatible with the host and where the eukaryotic cDNA coding for the receptor is inserted into the vector such that growth of the host containing the vector expresses the cDNA in

question. Usually, expression vectors are designed for stable replication in their host cells or for amplification to greatly increase the total number of copies of the desirable gene per cell. It is not always necessary to require that an expression vector replicate in a host cell, e.g., it is possible to effect transient expression of the protein or its fragments in various hosts using vectors that do not contain a replication origin that is recognized by the host cell. It is also possible to use vectors that cause integration of the protein encoding portion or its fragments into the host DNA by recombination.

Vectors, as used herein, comprise plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles which enable the integration of DNA fragments into the genome of the host. Expression vectors are specialized vectors which contain genetic control elements that effect expression of operably linked genes. Plasmids are the most commonly used form of vector but all other forms of vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein. See, e.g., Pouwels, et al., Cloning Vectors: A Laboratory Manual, Elsevier, N.Y. (1985), and Rodriquez, et al. (eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Buttersworth, Boston (1988), which are incorporated herein by reference.

Transformed cells are cells, preferably mammalian, that have been transformed or transfected with receptor vectors constructed using recombinant DNA techniques. Transformed host cells usually express the desired protein or its fragments, but for purposes of cloning, amplifying, and manipulating its DNA, do not need to express the subject protein. This invention further contemplates culturing transformed cells in a nutrient medium, thus permitting the receptor to accumulate in the cell membrane. The protein can be recovered, either from the culture or, in certain instances, from the culture medium.

For purposes of this invention, nucleic sequences are operably linked when they are functionally related to each other. For example, DNA for a presequence or secretory leader is operably linked to a polypeptide if it is expressed as a preprotein or participates in directing the polypeptide to the cell membrane or in secretion of the polypeptide. A promoter is operably linked to a coding sequence if it controls the transcription of the polypeptide; a ribosome binding site is operably linked to a coding sequence if it is positioned to permit translation. Usually, operably linked means contiguous and in reading frame, however, certain genetic elements such

as repressor genes are not contiguously linked but still bind to operator sequences that in turn control expression.

Suitable host cells include prokaryotes, lower eukaryotes, and higher eukaryotes. Prokaryotes include both gram negative and gram positive organisms, e.g., *E. coli* and *B. subtilis*. Lower eukaryotes include yeasts, e.g., *S. cerevisiae* and *Pichia*, and species of the genus Dictyostelium. Higher eukaryotes include established tissue culture cell lines from animal cells, both of non-mammalian origin, e.g., insect cells, and birds, and of mammalian origin, e.g., human, primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. As used herein, *E. coli* and its vectors will be used generically to include equivalent vectors used in other prokaryotes. A representative vector for amplifying DNA is pBR322 or many of its derivatives. Vectors that can be used to express the receptor or its fragments include, but are not limited to, such vectors as those containing the lac promoter (pUC-series); trp promoter (pBR322-trp); Ipp promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as ptac (pDR540). See Brosius, et al., "Expression Vectors Employing Lambda-, trp-, lac-, and Ipp-derived Promoters", in Vectors: A Survey of Molecular Cloning Vectors and Their Uses, (eds. Rodriguez and Denhardt), Buttersworth, Boston, Chapter 10, pp. 205-236 (1988), which is incorporated herein by reference.

Lower eukaryotes, e.g., yeasts and *Dictyostelium*, may be transformed with TLR sequence containing vectors. For purposes of this invention, the most common lower eukaryotic host is the baker's yeast, *Saccharomyces cerevisiae*. It will be used to generically represent lower eukaryotes although a number of other strains and species are also available. Yeast vectors typically consist of a replication origin (unless of the integrating type), a selection gene, a promoter, DNA encoding the receptor or its fragments, and sequences for translation termination, polyadenylation, and transcription termination. Suitable expression vectors for yeast include such constitutive promoters as 3-phosphoglycerate kinase and various other glycolytic enzyme gene promoters or such inducible promoters as the alcohol dehydrogenase 2 promoter or metallothionine promoter. Suitable vectors include derivatives of the following types: self-replicating low copy number (such as the YRp-series), self-replicating high copy number (such as the YEp-series); integrating types (such as the YIp-series), or mini-chromosomes (such as the YCp-series).

Higher eukaryotic tissue culture cells are normally the preferred host cells for expression of the functionally active interleukin protein. In principle, any higher eukaryotic tissue culture cell line is workable, e.g., insect baculovirus expression systems, whether from an invertebrate or vertebrate source. However, mammalian cells are preferred. Transformation or transfection and propagation of such cells has become a routine procedure. Examples of useful cell lines include HeLa cells, Chinese hamster ovary (CHO) cell lines, baby rat kidney (BRK) cell lines, insect cell lines, bird cell lines, and monkey (COS) cell lines. Expression vectors for such cell lines usually include an origin of replication, a promoter, a translation initiation site, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a transcription termination site. These vectors also usually contain a selection gene or amplification gene. Suitable expression vectors may be plasmids, viruses, or retroviruses carrying promoters derived, e.g., from such sources as from adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus. Representative examples of suitable expression vectors include pCDNA1; pCD, see Okayama, et al., Mol. Cell Biol. 5, 1136 (1985); pMC1neo PolyA, see Thomas, et al., Cell 51, 503 (1987); and a baculovirus vector such as pAC 373 or pAC 610.

For secreted proteins, an open reading frame usually encodes a polypeptide that consists of a mature or secreted product covalently linked at its N-terminus to a signal peptide. The signal peptide is cleaved prior to secretion of the mature, or active, polypeptide. The cleavage site can be predicted with a high degree of accuracy from empirical rules, e.g., von-Heijne, Nucleic Acids Research 14, 4683 (1986), and the precise amino acid composition of the signal peptide does not appear to be critical to its function, e.g., Randall, et al., Science 243, 1156 (1989); Kaiser, et al., Science 235, 312 (1987).

It will often be desired to express these polypeptides in a system which provides a specific or defined glycosylation pattern. In this case, the usual pattern will be that provided naturally by the expression system. However, the pattern will be modifiable by exposing the polypeptide, e.g., an unglycosylated form, to appropriate glycosylating proteins introduced into a heterologous expression system. For example, the receptor gene may be co-transformed with one or more genes encoding mammalian or other glycosylating enzymes. Using this approach, certain mammalian glycosylation patterns will be achievable in prokaryote or other cells.

The source of TLR can be a eukaryotic or prokaryotic host expressing recombinant TLR, such as is described above. The source can also be a cell line such as mouse Swiss 3T3

fibroblasts, but other mammalian cell lines are also contemplated by this invention, with the preferred cell line being from the human species.

Now that the sequences are known, the primate TLRs, fragments, or derivatives thereof can be prepared by conventional processes for synthesizing peptides. These include processes such as are described in Stewart and Young, Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL (1984); Bodanszky and Bodanszky, The Practice of Peptide Synthesis, Springer-Verlag, New York; and Bodanszky (1984) The Principles of Peptide Synthesis, Springer-Verlag, New York; all of each which are incorporated herein by reference. For example, an azide process, an acid chloride process, an acid anhydride process, a mixed anhydride process, an active ester process (e.g., p-nitrophenyl ester, N-hydroxysuccinimide ester, or cyanomethyl ester), a carbodiimidazole process, an oxidative-reductive process, or a dicyclohexylcarbodiimide (DCCD)/additive process can be used. Solid phase and solution phase syntheses are both applicable to the foregoing processes. Similar techniques can be used with partial TLR sequences.

The TLR proteins, fragments, or derivatives are suitably prepared in accordance with the above processes as typically employed in peptide synthesis, generally either by a so-called stepwise process which comprises condensing an amino acid to the terminal amino acid, one by one in sequence, or by coupling peptide fragments to the terminal amino acid. Amino groups that are not being used in the coupling reaction typically must be protected to prevent coupling at an incorrect location.

If a solid phase synthesis is adopted, the C-terminal amino acid is bound to an insoluble carrier or support through its carboxyl group. The insoluble carrier is not particularly limited as long as it has a binding capability to a reactive carboxyl group. Examples of such insoluble carriers include halomethyl resins, such as chloromethyl resin or bromomethyl resin, hydroxymethyl resins, phenol resins, tert-alkyloxycarbonylhydrazidated resins, and the like.

An amino group-protected amino acid is bound in sequence through condensation of its activated carboxyl group and the reactive amino group of the previously formed peptide or chain, to synthesize the peptide step by step. After synthesizing the complete sequence, the peptide is split off from the insoluble carrier to produce the peptide. This solid-phase approach is generally described by Merrifield, et al., J. Am. Chem. Soc. 85, 2149 (1963), which is incorporated herein by reference.

The prepared protein and fragments thereof can be isolated and purified from the reaction mixture by means of peptide separation, for example, by extraction, precipitation, electrophoresis, various forms of chromatography, and the like. The receptors of this invention can be obtained in varying degrees of purity depending upon desired uses. Purification can be accomplished by use of the protein purification techniques disclosed herein, see below, or by the use of the antibodies herein described in methods of immunoabsorbant affinity chromatography. This immunoabsorbant affinity chromatography is carried out by first linking the antibodies to a solid support and then contacting the linked antibodies with solubilized lysates of appropriate cells, lysates of other cells expressing the receptor, or lysates or supernatants of cells producing the protein as a result of DNA techniques, see below.

Generally, the purified protein will be at least about 40% pure, ordinarily at least about 50% pure, usually at least about 60% pure, typically at least about 70% pure, more typically at least about 80% pure, preferable at least about 90% pure and more preferably at least about 95% pure, and in particular embodiments, 97%-99% or more. Purity will usually be on a weight basis, but can also be on a molar basis. Different assays will be applied as appropriate.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of a TLR, and to variants of a TLR polypeptide. Variants may occur naturally, such as a ***natural allelic variant.*** By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism (Lewin, Genes II, John Wiley and Sons, New York (1985)). Non-naturally occurring variants may be produced using art-known mutagenesis techniques. Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Some contemplated examples of conservative substitutions include substitution of a hydrophobic residue such as isoleucine, valine, leucine or methionine for another hydrophobic residue. Also, a polar residue such as arginine, lysine, glutamic acid, aspartic acid, glutamine, asparagine, and the like, can be conservatively substituted for another member of this group. Still another aspect of a polypeptide incorporating conservative substitutions occurs when a substituted amino acid residue replaces an unsubstituted parent amino acid residue. The variations may include silent

substitutions, additions and deletions, which do not alter the properties and activities of the TLR or portions thereof.

VI. Antibodies

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies, antibody compositions with polyepitopic specificity, bispecific antibodies, and single-chain molecules, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv), so long as they antagonize the biological activity of TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, or TLR10.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., Nature, 256: 495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., Nature, 352, 624(1991) and Marks et al., J. Mol. Biol., 222, 581(1991), for example.

Monoclonal antibodies include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another

antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., Proc. Natl. Acad. Sci. USA, 81, 6851(1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., Nature, 321, 522 (1986); Reichmann et al., Nature, 332, 323(1988); and Presta, Curr. Op. Struct. Biol., 2, 593 (1992). The humanized antibody includes a Primatized.TM. antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315(1994).

Antibodies can be raised to the various TLR proteins and fragments thereof, both in naturally occurring native forms and in their recombinant forms, the difference being that antibodies to the active receptor are more likely to recognize epitopes which are only present in the native conformations. Denatured antigen detection can also be useful in, e.g., Western analysis.

A TLR of this invention can be used as an immunogen for the production of antisera or antibodies specific, e.g., capable of distinguishing between various Toll-like receptors or various fragments thereof. The purified TLR can be used to screen monoclonal antibodies or antigen-binding fragments prepared by immunization with various forms of impure preparations containing the protein.

The purified TLR can also be used as a reagent to detect antibodies generated in response to the presence of elevated levels of expression, or immunological disorders which lead to antibody production to the endogenous receptor.

Additionally, TLR fragments may also serve as immunogens to produce the antibodies of the present invention, as described immediately below. For example, this invention contemplates antibodies having binding affinity to or being raised against the amino acid sequences shown in SEQ ID NOS: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 37, 39, 41, 43, or 45, fragments thereof, or various homologous peptides. In particular, this invention contemplates antibodies having binding affinity to, or having been raised against, specific fragments which are predicted to be, or actually are, exposed at the exterior protein surface of the native TLR.

The blocking of physiological response to the receptor ligands may result from the inhibition of binding of the ligand to the receptor, likely through competitive inhibition. Thus, *in vitro* assays of the present invention will often use antibodies or antigen binding segments of these antibodies, or fragments attached to solid phase substrates. These assays will also allow for the diagnostic determination of the effects of either ligand binding region mutations and modifications, or other mutations and modifications, e.g., which affect signaling or enzymatic function.

This invention also contemplates the use of competitive drug screening assays, e.g., where antibodies to the receptor (or antibody fragments) compete with a test compound for binding to a ligand or other antibody. The invention also contemplates use of water-soluble

versions of the Toll-like receptors for drug screening. In this manner, the neutralizing antibodies or fragments can be used to detect the presence of a polypeptide which shares one or more binding sites to a receptor and can also be used to occupy binding sites on a receptor that might otherwise bind a ligand.

Preferred antibodies will exhibit properties of both affinity and selectivity. High affinity is generally preferred, while selectivity will allow distinction between various embodiment subsets. In particular, it will be desirable to possess antibody preparations characterized to bind, e.g., various specific combinations of related members while not binding others. Such various combinatorial subsets are specifically enabled, e.g., these reagents may be generated or selected using standard methods of immunoaffinity, selection, etc.

Antibodies, including binding fragments and single chain versions, against predetermined fragments of the protein can be raised by immunization of animals with conjugates of the fragments with immunogenic proteins. Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies can be screened for binding to normal or defective protein, or screened for agonistic or antagonistic activity. These monoclonal antibodies will usually bind with at least a K_D of about 1 mM, more usually at least about 300 μM, typically at least about 100μM, more typically at least about 30 μM, preferably at least about 10 μM, and more preferably at least about 3 μM or better.

The antibodies, including antigen binding fragments, of this invention can have significant diagnostic or therapeutic value. They can be potent antagonists that bind to the receptor and inhibit binding to ligand or inhibit the ability of the receptor to elicit a biological response, e.g., act on its substrate. They can also be agonists that bind to the receptor, and initiate signals that are similar to those stimulated to the receptor's ligand under physiological conditions. Antibodies to a Toll-like receptor can also be coupled to toxins or radionuclides to produce a conjugate, where the conjugate can be used for inhibiting or killing cells bearing a Toll-like receptor. Further, these antibodies can be conjugated to drugs or other therapeutic agents, either directly or indirectly by means of a linker.

The antibodies of this invention can also be useful in diagnostic applications. As capture or non-neutralizing antibodies, they might bind to the receptor without inhibiting ligand or substrate binding. As neutralizing antibodies, they can be useful in competitive binding assays. They will also be useful in detecting or quantifying ligand. They may be used as reagents for

Western blot analysis, or for immunoprecipitation or immunopurification of the respective protein.

Protein fragments may be joined to other materials, particularly polypeptides, as fused or covalently joined polypeptides to be used as immunogens. Mammalian TLR and its fragments may be fused or covalently linked to a variety of immunogens, such as keyhole limpet hemocyanin, bovine serum albumin, tetanus toxoid, etc. See Microbiology, Hoeber Medical Division, Harper and Row, (1969); Landsteiner, Specificity of Serological Reactions, Dover Publications, New York (1962); and Williams, et al., Methods in Immunology and Immunochemistry, Vol. 1, Academic Press, New York (1967); each of which are incorporated herein by reference, for descriptions of methods of preparing polyclonal antisera. A typical method involves hyperimmunization of an animal with an antigen. The blood of the animal is then collected shortly after the repeated immunizations and the gamma globulin is isolated.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such monoclonal antibodies may be found in, e.g., Stites, et al. (eds.), Basic and Clinical Immunology (4th ed.), Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH Press; Goding, Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York (1986); and particularly in Kohler and Milstein, Nature 256, 495 (1975), which discusses one method of generating monoclonal antibodies. Each of these references is incorporated herein by reference. Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Other suitable techniques involve *in vitro* exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See, Huse, et al., Science 246, 1275 (1989); and Ward, et al., Nature 341, 544 (1989), each of which is hereby incorporated herein by reference. The polypeptides and antibodies of the

present invention may be used with or without modification, including chimeric or humanized antibodies. Frequently, the polypeptides and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents, teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant or chimeric immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567; or made in transgenic mice, see Mendez, et al., Nature Genetics 15, 146 (1997). These references are incorporated herein by reference.

The antibodies of this invention can also be used for affinity chromatography in isolating the TLRs. Columns can be prepared where the antibodies are linked to a solid support, e.g., particles, such as AGAROSE, SEPHADEX, or the like, where a cell lysate may be passed through the column, the column washed, followed by increasing concentrations of a mild denaturant, whereby the purified protein will be released. The protein may be used to purify antibody.

The antibodies may also be used to screen expression libraries for particular expression products. Usually the antibodies used in such a procedure will be labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against a TLR will also be used to raise anti-idiotypic antibodies. These will be useful in detecting or diagnosing various immunological conditions related to expression of the protein or cells which express the protein. They also will be useful as agonists or antagonists of the ligand, which may be competitive inhibitors or substitutes for naturally occurring ligands.

A TLR protein that specifically binds to or that is specifically immunoreactive with an antibody generated against a defined immunogen, such as an immunogen consisting of the amino acid sequence of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24, is typically determined in an immunoassay. The immunoassay typically uses a polyclonal antiserum which was raised, e.g., to a protein of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24. This antiserum is selected to have low crossreactivity against other IL-1R family members, e.g., TLR1, preferably

from the same species, and any such crossreactivity is removed by immunoabsorption prior to use in the immunoassay.

In order to produce antisera for use in an immunoassay, the protein of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24, or a combination thereof, is isolated as described herein. For example, recombinant protein may be produced in a mammalian cell line. An appropriate host, e.g., an inbred strain of mice such as Balb/c, is immunized with the selected protein, typically using a standard adjuvant, such as Freund's adjuvant, and a standard mouse immunization protocol (see Harlow and Lane, supra). Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used an immunogen. Polyclonal sera are collected and titered against the immunogen protein in an immunoassay, e.g., a solid phase immunoassay with the immunogen immobilized on a solid support. Polyclonal antisera with a titer of 10^4 or greater are selected and tested for their cross reactivity against other IL-1R family members, e.g., mouse TLRs or human TLR1, using a competitive binding immunoassay such as the one described in Harlow and Lane, supra, at pages 570-573. Preferably at least two TLR family members are used in this determination in conjunction with either or some of the human TLR2-10. These IL-1R family members can be produced as recombinant proteins and isolated using standard molecular biology and protein chemistry techniques as described herein.

Immunoassays in the competitive binding format can be used for the crossreactivity determinations. For example, the proteins of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and/or 24, or various fragments thereof, can be immobilized to a solid support. Proteins added to the assay compete with the binding of the antisera to the immobilized antigen. The ability of the above proteins to compete with the binding of the antisera to the immobilized protein is compared to the protein of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and/or 24. The percent crossreactivity for the above proteins is calculated, using standard calculations. Those antisera with less than 10% crossreactivity with each of the proteins listed above are selected and pooled. The cross-reacting antibodies are then removed from the pooled antisera by immunoabsorption with the above-listed proteins.

The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay as described above to compare a second protein to the immunogen protein (e.g., the IL-1R like protein of SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and/or 24). In order to

make this comparison, the two proteins are each assayed at a wide range of concentrations and the amount of each protein required to inhibit 50% of the binding of the antisera to the immobilized protein is determined. If the amount of the second protein required is less than twice the amount of the protein of the selected protein or proteins that is required, then the second protein is said to specifically bind to an antibody generated to the immunogen.

It is understood that these TLR proteins are members of a family of homologous proteins that comprise at least 10 so far identified genes. For a particular gene product, such as the TLR2-10, the term refers not only to the amino acid sequences disclosed herein, but also to other proteins that are allelic, non-allelic or species variants. It also understood that the terms include nonnatural mutations introduced by deliberate mutation using conventional recombinant technology such as single site mutation, or by excising short sections of DNA encoding the respective proteins, or by substituting new amino acids, or adding new amino acids. Such minor alterations must substantially maintain the immunoidentity of the original molecule and/or its biological activity. Thus, these alterations include proteins that are specifically immunoreactive with a designated naturally occurring IL-1R related protein, for example, the TLR proteins shown in SEQ ID NO: 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24. The biological properties of the altered proteins can be determined by expressing the protein in an appropriate cell line and measuring the appropriate effect upon lymphocytes. Particular protein modifications considered minor would include conservative substitution of amino acids with similar chemical properties, as described above for the IL-1R family as a whole. By aligning a protein optimally with the protein of TLR2-10 and by using the conventional immunoassays described herein to determine immunoidentity, one can determine the protein compositions of the invention.

VII. Kits and quantitation

Both naturally occurring and recombinant forms of the IL-1R like molecules of this invention are particularly useful in kits and assay methods. For example, these methods would also be applied to screening for binding activity, e.g., ligands for these proteins. Several methods of automating assays have been developed in recent years so as to permit screening of tens of thousands of compounds per year. See, e.g., a BIOMEK automated workstation, Beckman Instruments, Palo Alto, California, and Fodor, et al., Science 251, 767 (1991), which is incorporated herein by reference. The latter describes means for testing binding by a plurality of

defined polymers synthesized on a solid substrate. The development of suitable assays to screen for a ligand or agonist/antagonist homologous proteins can be greatly facilitated by the availability of large amounts of purified, soluble TLRs in an active state such as is provided by this invention.

Purified TLR can be coated directly onto plates for use in the aforementioned ligand screening techniques. However, non-neutralizing antibodies to these proteins can be used as capture antibodies to immobilize the respective receptor on the solid phase, useful, e.g., in diagnostic uses.

This invention also contemplates use of TLR2-10, fragments thereof, peptides, and their fusion products in a variety of diagnostic kits and methods for detecting the presence of the protein or its ligand. Alternatively, or additionally, antibodies against the molecules may be incorporated into the kits and methods. Typically the kit will have a compartment containing either a defined TLR peptide or gene segment or a reagent which recognizes one or the other. Typically, recognition reagents, in the case of peptide, would be a receptor or antibody, or in the case of a gene segment, would usually be a hybridization probe.

A preferred kit for determining the concentration of, e.g., TLR4, a sample would typically comprise a labeled compound, e.g., ligand or antibody, having known binding affinity for TLR4, a source of TLR4 (naturally occurring or recombinant) as a positive control, and a means for separating the bound from free labeled compound, for example a solid phase for immobilizing the TLR4 in the test sample. Compartments containing reagents, and instructions, will normally be provided.

Antibodies, including antigen binding fragments, specific for mammalian TLR or a peptide fragment, or receptor fragments are useful in diagnostic applications to detect the presence of elevated levels of ligand and/or its fragments. Diagnostic assays may be homogeneous (without a separation step between free reagent and antibody-antigen complex) or heterogeneous (with a separation step). Various commercial assays exist, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), enzyme-multiplied immunoassay technique (EMIT), substrate-labeled fluorescent immunoassay (SLFIA) and the like. For example, unlabeled antibodies can be employed by using a second antibody which is labeled and which recognizes the antibody to TLR4 or to a particular fragment thereof. These assays have also been extensively discussed in the literature.

See, e.g., Harlow and Lane, Antibodies: A Laboratory Manual, CSH. (1988), and Coligan, Current Protocols In Immunology, Greene/Wiley, New York (1991).

Anti-idiotypic antibodies may have similar use to serve as agonists or antagonists of TLR4. These should be useful as therapeutic reagents under appropriate circumstances.

Frequently, the reagents for diagnostic assays are supplied in kits, so as to optimize the sensitivity of the assay. For the subject invention, depending upon the nature of the assay, the protocol, and the label, either labeled or unlabeled antibody, or labeled ligand is provided. This is usually in conjunction with other additives, such as buffers, stabilizers, materials necessary for signal production such as substrates for enzymes, and the like. Preferably, the kit will also contain instructions for proper use and disposal of the contents after use. Typically the kit has compartments for each useful reagent, and will contain instructions for proper use and disposal of reagents. Desirably, the reagents are provided as a dry lyophilized powder, where the reagents may be reconstituted in an aqueous medium having appropriate concentrations for performing the assay.

The aforementioned constituents of the diagnostic assays may be used without modification or may be modified in a variety of ways. For example, labeling may be achieved by covalently or non-covalently joining a moiety which directly or indirectly provides a detectable signal. In any of these assays, a test compound, TLR, or antibodies thereto can be labeled either directly or indirectly. Possibilities for direct labeling include label groups: radiolabels such as ^{125}I, enzymes (U.S. Pat. No. 3,645,090) such as peroxidase and alkaline phosphatase, and fluorescent labels (U.S. Pat. No. 3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization. Both of the patents are incorporated herein by reference. Possibilities for indirect labeling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups.

There are also numerous methods of separating the bound from the free ligand, or alternatively the bound from the free test compound. The TLR can be immobilized on various matrixes followed by washing. Suitable matrices include plastic such as an ELISA plate, filters, and beads. Methods of immobilizing the receptor to a matrix include, without limitation, direct adhesion to plastic, use of a capture antibody, chemical coupling, and biotin-avidin. The last step in this approach involves the precipitation of antibody/antigen complex by any of several methods including those utilizing, e.g., an organic solvent such as polyethylene glycol or a salt

such as ammonium sulfate. Other suitable separation techniques include, without limitation, the fluorescein antibody magnetizable particle method described in Rattle, et al., Clin. Chem. 30, 1457 (1984), and the double antibody magnetic particle separation as described in U.S. Pat. No. 4,659,678, each of which is incorporated herein by reference.

The methods for linking protein or fragments to various labels have been extensively reported in the literature and do not require detailed discussion here. Many of the techniques involve the use of activated carboxyl groups either through the use of carbodiimide or active esters to form peptide bonds, the formation of thioethers by reaction of a mercapto group with an activated halogen such as chloroacetyl, or an activated olefin such as maleimide, for linkage, or the like. Fusion proteins will also find use in these applications.

Another diagnostic aspect of this invention involves use of oligonucleotide or polynucleotide sequences taken from the sequence of a TLR. These sequences can be used as probes for detecting levels of the respective TLR in patients suspected of having an immunological disorder. The preparation of both RNA and DNA nucleotide sequences, the labeling of the sequences, and the preferred size of the sequences has received ample description and discussion in the literature. Normally an oligonucleotide probe should have at least about 14 nucleotides, usually at least about 18 nucleotides, and the polynucleotide probes may be up to several kilobases. Various labels may be employed, most commonly radionuclides, particularly ^{32}P. However, other techniques may also be employed, such as using biotin modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed which can recognize specific duplexes, including DNA duplexes, RNA duplexes, DNA-RNA hybrid duplexes, or DNA-protein duplexes. The antibodies in turn may be labeled and the assay carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected. The use of probes to the novel anti-sense RNA may be carried out in any conventional techniques such as nucleic acid hybridization, plus and minus screening, recombinational probing, hybrid released translation (HRT), and hybrid arrested translation (HART). This also includes amplification techniques such as polymerase chain reaction (PCR).

Diagnostic kits which also test for the qualitative or quantitative presence of other markers are also contemplated. Diagnosis or prognosis may depend on the combination of multiple indications used as markers. Thus, kits may test for combinations of markers. See, e.g., Viallet, et al., Progress in Growth Factor Res. 1, 89 (1989).

VIII. Therapeutic Utility

This invention provides reagents with significant therapeutic value. The TLRs (naturally occurring or recombinant), fragments thereof, mutein receptors, and antibodies, along with compounds identified as having binding affinity to the receptors or antibodies, should be useful in the treatment of conditions exhibiting abnormal expression of the receptors of their ligands. Such abnormality will typically be manifested by immunological disorders. Additionally, this invention should provide therapeutic value in various diseases or disorders associated with abnormal expression or abnormal triggering of response to the ligand. The Toll ligands have been suggested to be involved in morphologic development, e.g., dorso-ventral polarity determination, and immune responses, particularly the primitive innate responses. See, e.g., Sun, et al., Eur. J. Biochem. 196, 247 (1991); Hultmark, Nature 367, 116 (1994).

Recombinant TLRs, muteins, agonist or antagonist antibodies thereto, or antibodies can be purified and then administered to a patient. These reagents can be combined for therapeutic use with additional active ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, along with physiologically innocuous stabilizers and excipients. These combinations can be sterile, e.g., filtered, and placed into dosage forms as by lyophilization in dosage vials or storage in stabilized aqueous preparations. This invention also contemplates use of antibodies or binding fragments thereof which are not complement binding.

Ligand screening using TLR or fragments thereof can be performed to identify molecules having binding affinity to the receptors. Subsequent biological assays can then be utilized to determine if a putative ligand can provide competitive binding, which can block intrinsic stimulating activity. Receptor fragments can be used as a blocker or antagonist in that it blocks the activity of ligand. Likewise, a compound having intrinsic stimulating activity can activate the receptor and is thus an agonist in that it simulates the activity of ligand, e.g., inducing signaling. This invention further contemplates the therapeutic use of antibodies to TLRs as antagonists.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used in vitro may provide useful guidance in the amounts useful for in situ administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman, et al., Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press (1990); which is hereby incorporated herein by reference. Methods for administration are discussed therein and below, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the Merck Index, Merck & Co., Rahway, New Jersey. Because of the likely high affinity binding, or turnover numbers, between a putative ligand and its receptors, low dosages of these reagents would be initially expected to be effective. And the signaling pathway suggests extremely low amounts of ligand may have effect. Thus, dosage ranges would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 μM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Slow release formulations, or slow release apparatus will often be utilized for continuous administration.

TLRs, fragments thereof, and antibodies or its fragments, antagonists, and agonists, may be administered directly to the host to be treated or, depending on the size of the compounds, it may be desirable to conjugate them to carrier proteins such as ovalbumin or serum albumin prior to their administration. Therapeutic formulations may be administered in any conventional dosage formulation. While it is possible for the active ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation. Formulations comprise at least one active ingredient, as defined above, together with one or more acceptable carriers thereof. Each carrier must be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration. The formulations may conveniently be presented in

unit dosage form and may be prepared by any methods well known in the art of pharmacy. See, e.g., Gilman, et al., Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press (1990); and Avis, et al., Pharmaceutical Dosage Forms: Parenteral Medications, Dekker, NY (1993); Lieberman, et al., Pharmaceutical Dosage Forms: Tablets Dekker, NY (1990); and Lieberman, et al., Pharmaceutical Dosage Forms: Disperse Systems Dekker, NY (1990). The therapy of this invention may be combined with or used in association with other therapeutic agents, particularly agonists or antagonists of other IL-1 family members.

IX. Ligands

The description of the Toll-like receptors herein provide means to identify ligands, as described above. Such ligand should bind specifically to the respective receptor with reasonably high affinity. Various constructs are made available which allow either labeling of the receptor to detect its ligand. For example, directly labeling TLR, fusing onto it markers for secondary labeling, e.g., FLAG or other epitope tags, etc., will allow detection of receptor. This can be histological, as an affinity method for biochemical purification, or labeling or selection in an expression cloning approach. A two-hybrid selection system may also be applied making appropriate constructs with the available TLR sequences. See, e.g., Fields and Song, Nature 340, 245 (1989).

Generally, descriptions of TLRs will be analogously applicable to individual specific embodiments directed to TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and/or TLR10 reagents and compositions.

The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the inventions to the specific embodiments.

X. Isolation and culture of cells.

Blood $CD11C^+$ immature dendritic cells, plasmacytoid pre-dendritic cells, and $CD14^+CD16^-$ monocytes were isolated from human peripheral blood, according to Rissoan, et al., Science 283, 1183 (1999) and Grouard, et al., J. Exp. Med. 185, 1101 (1997). The purity of each cell population was over 99%. Monocytes were cultured for five days in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.055 mM 2-

mercaptoethanol, penicillin G, and streptomycin (Invitrogen Life Technologies, Carlsbad, CA), in the presence of 50 ng/ml GM-CSF (Schering-Plough, Kenilworth, NJ)and 200 U/ml IL-4 (Schering-Plough, Kenilworth, NJ). The resulting monocyte-derived immature dendritic cells were washed and cultured for 24 h with human CD40L-transfected L cells (irratiated at 5,500 rad) to obtain mature dendric cells type 1 (Rissoan, et al., Science 283, 1183 (1999)). Plasmacytoid pre-dendritic cells were cultured for five days with 10 ng/ml IL-3 (R & D Systems). The resulting plasmacytoid pre-dendritic cells-derived immature dendritic cells were washed and cultured for 24 h, with CD40L-transfected cells to obtain pre-dendritic cell-derived dendritic cells. To induce the maturation of immature dendritic cells, the cells were cultured for 24 h with CD40L-transfected L cells.

To induce cytokine production, cells were cultured for 24 h at two times 10^4/0.2 ml in round-bottom 96-well culture plates in the presence of 0.01 mg/ml peptidoglycan from S. aureus (Fluka, Milwaukee, WI), 0.01 mg/ml lipoteichoic acid (LTA) from S. aureus (Sigma, St. Louis, MO), 0.01 mg/ml LPS from *S. minnesota* serotype Re595 (Sigma, St. Louis, MO), 0.05 mg/ml Poly I:C (Sigma, St. Louis, MO), 0.005 mM (0.046 mg/ml) phosphodiester CpG oligodeoxynucleotide (AAC-30) (Yamamoto, et al, Jpn. J. Cancer Res. 85, 775 (1994)). AAC-30 was added at 0, 4, and 16 h to compensate for degradation by DNase activity in the medium.

XI. Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction for the detection of mRNA coding for Toll-like receptors was as follows. RNA was isolated with the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, Anal. Biochem. 162, 156 (1987)). Contaminating DNA was removed by digestion with 5 U deoxyribonuclease I (Boehringer Mannheim) for 30 min at 37°C. Reverse transcription was carried out with random hexamers (Promega, Madison, WI) for priming and SUPERSCRIPT II (Invitrogen Life Technologies, Carlsbad, CA). The PCR reaction volume was 0.05 ml, containing 0.5 μM of each primer, 40 nM of each deoxynucleoside triphosphate, and 1.25 U AMPLITAQ (Perkin Elmer, Foster City, CA). Primers used are shown in Table 1.

Table 1. Sequences of PCR primers.

Reverse transcriptase PCR primers.

Forward primers/Reverse primers

TLR#	
1	CGTAAAACTGGAAGCTTTGCAAGA
	CCTTGGGCCATTCCAAATAAGTCC
2	GGCCAGCAAATTACCTGTGTG
	CCAGGTAGGTCTTGGTGTTCA
3	ATTGGGTCTGGGAACATTTCTCTTC
	GTGAGATTTAAACATTCCTCTTCGC
4	CTGCAATGGATCAAGGACCA
	TCCCACTCCAGGTAAGTGTT
5	CATTGTATGCACTGTCACTC
	CCACCACCATGATGAGAGCA
6	TAGGTCTCATGACGAAGGAT
	GGCCACTGCAAATAAGTCCG
7	AGTGTCTAAAGAACCTGG
	CTTGGCCTTACAGAAATG
8	CAGAATAGCAGGCGTAACACATCA
	AATGTCACAGGTGCATTCAAAGGG
9	TTATGGACTTCCTGCTGGAGGTGC
	CTGCGTTTTGTCGAAGACCA
10	CAATCTAGAGAAGGAAGATGGTCC
	GCCCTTATAAACTTGTGAAGGTGT
β-actin	
	ATCTGGCACCACACCTTCTACAATGAGCTGCG
	CGTCATACTCCTGCTTGCTGATCCACATCTGC

Real time PCR primers.

Forward primers/Reverse primers

Toll like receptor	
2	GGCCAGCAAATTACCTGTGTG
	AGGCGGACATCCTGAACCT

4	CTGCAATGGATCAAGGACCA
	TTATCTGAAGGTGTTGCACATTCC
7	TTACCTGGATGGAAACCAGCTACT
	TCAAGGCTGAGAAGCTGTAAGCTA
9	TGAAGACTTCAGGCCCAACTG
	TGCACGGTCACCAGGTTGT

A GENEAMP PCR System 9700 (Perkin Elmer/Applied Biosystems, Foster City, CA) was used with an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and a final elongation step of 72°C for 7 min. PCR products were separated on a 3% agarose gel containing ethidium bromide. A 1-kb DNA ladder standard (Invitrogen Life Technologies, Carlsbad, CA) was used as a size marker.

XII. Real-time quantitative reverse transcription PCR

RNA was isolated with the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, Anal. Biochem. 162, 156 (1987)). The reverse transcription was performed with SUPERSCRIPT II (Invitrogen Life Technologies, Carlsbad, CA). cDNA was analyzed for the expression of Toll like receptor genes by the fluorogenic 5'-nuclease PCR assay (Rissoan, et al., Science 283, 1183 (1999)) using a Perkin-Elmer ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Reactions were incubated for 2 min at 50°C, denatured for 10 min at 95°C, and subjected to 40 two-step amplification cycles with annealing/extension at 60°C for 1 min, followed by denaturation at 95°C for 15 sec. The primers used are shown in Table 1. Values are expressed as arbitrary units (relative to ubiquitin X 1,000).

XIII. Quantitation of cytokines by ELISA

ELISA kits from the following companies were used to analyze cytokine production: TNF-α and IL-6 (R & D Systems, Minneapolis, MN), IL-12 and IFN-α (Biosource International, Camarillo, CA).

EXAMPLES

EXAMPLE I. General Methods

Some of the standard methods are described or referenced, e.g., in Maniatis, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press (1982); Sambrook, et al., Molecular Cloning: A Laboratory Manual, (2d ed.), vols. 1-3, CSH Press, NY (1989); Ausubel, et al., Current Protocols in Molecular Biology, Greene/Wiley, New York (1987). Methods for protein purification include such methods as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Deutscher, "Guide to Protein Purification" in Methods in Enzymology, vol. 182 (1990), and other volumes in this series; and manufacturer's literature on use of protein purification products, e.g., Pharmacia, Piscataway, N.J., or Bio-Rad, Richmond, CA. Combination with recombinant techniques allow fusion to appropriate segments, e.g., to a FLAG sequence or an equivalent which can be fused via a protease-removable sequence. See, e.g., Hochuli, Chemische Industrie 12, 69 (1989); Hochuli, "Purification of Recombinant Proteins with Metal Chelate Absorbent" in Setlow (ed.) Genetic Engineering, Principle and Methods 12, 87 (1990), Plenum Press, N.Y.; and Crowe, et al., QIAexpress: The High Level Expression and Protein Purification System QUIAGEN, Inc., Chatsworth, CA (1992).

Standard immunological techniques and assays are described, e.g., in Hertzenberg, et al., Weir's Handbook of Experimental Immunology vols. 1-4, Blackwell Science (1996); Coligan (1991) Current Protocols in Immunology Wiley/Greene, NY; and Methods in Enzymology volumes. 70, 73, 74, 84, 92, 93, 108, 116, 121, 132, 150, 162, and 163.

Assays for vascular biological activities are well known in the art. They will cover angiogenic and angiostatic activities in tumor, or other tissues, e.g., arterial smooth muscle proliferation (see, e.g., Koyoma, et al., Cell 87, 1069 (1996), monocyte adhesion to vascular epithelium (see McEvoy, et al., J. Exp. Med. 185:2069 (1997), Ross, Nature 362, 801 (1993); Rekhter and Gordon, Am. J. Pathol. 147, 668 (1995); Thyberg, et al., Atherosclerosis 10, 966 (1990); and Gumbiner, Cell 84, 345 (1996).

Assays for neural cell biological activities are described, e.g., in Wouterlood, Neuroscience Protocols modules 10, Elsevier; Methods in Neurosciences Academic Press (1995); and Neuromethods Humana Press, Totowa, NJ. Methodology of developmental systems

is described in Meisami, Handbook of Human Growth and Developmental Biology CRC Press (1988).

Computer sequence analysis is performed, e.g., using available software programs, including the GCG WISCONSIN PACKAGE (Accelrys, Inc., San Diego, CA). Public sequence databases were also used, e.g., from GenBank, NCBI, EMBO, and others. Determination of transmembrane and other important motifs may be predicted using such bioinformatics tools.

Many techniques that have been used, as they relate to interleukin-10 receptors, may also be applied to the Toll-like receptors, e.g., U.S. Patent Nos. 5,789,192, issued to Moore, et al., 5,985,828, issued to Moore, et al., and 5,863,796, issued to Moore, et al., which are incorporated herein by reference for all purposes.

EXAMPLE II. Novel Family of Human Receptors

The discovery of sequence homology between the cytoplasmic domains of Drosophila Toll and human interleukin-1 (IL-1) receptors suggests that both molecules are used in signaling pathways that involve Rel-type transcription factors. This conserved signaling scheme governs an evolutionarily ancient immune response in both insects and vertebrates. We report the molecular cloning of a novel class of putative human receptors with a protein architecture that is closely similar to Drosophila Toll in both intra- and extra-cellular segments. Five human Toll-like receptors, designated TLRs 1-5, are likely the direct homologs of the fly molecule, and as such could constitute an important and unrecognized component of innate immunity in humans; intriguingly, the evolutionary retention of TLRs in vertebrates may indicate another role, akin to Toll in the dorso-ventralization of the Drosophila embryo, as regulators of early morphogenetic patterning. Multiple tissue mRNA blots indicate markedly different patterns of expression for the human TLRs. Using fluorescence in situ hybridization and Sequence-Tagged Site database analyses, we also show that the cognate TLR genes reside on chromosomes 4 (TLRs 1, 2, and 3), 9 (TLR4), and 1 (TLR5). Structure prediction of the aligned Toll-homology (TH) domains from varied insect and human TLRs, vertebrate IL-1 receptors, and MyD88 factors, and plant disease resistance proteins, recognizes a parallel β/α fold with an acidic active site; a similar structure notably recurs in a class of response regulators broadly involved in transducing sensory information in bacteria.

The study of the Toll receptors of invertebrates and the Toll-like receptors of mammal, has revealed a family of receptors and signaling pathways that has been maintained during evolution (DeRobertis and Sasai, Nature 380, 37 (1996); Arendt and Nübler-Jung, Mech. Develop. 61, 7 (1997); Miklos and Rubin, Cell 86, 521 (1996); Chothia, Develop. 1994 Suppl., 27 (1994); Banfi, et al., Nature Genet. 13, 167 (1996)). The study of the Toll-like receptors, as they are used in the mammalian immune system and mammalian development, may be made easier by a knowlege of the role of these receptors in more primitive animals.

A universally critical step in embryonic development is the specification of body axes, either born from innate asymmetries or triggered by external cues (DeRobertis and Sasai, Nature 380, 37 (1996); Arendt and Nübler-Jung, Mech. Develop. 61, 7 (1997)). As a model system, particular attention has been focused on the phylogenetic basis and cellular mechanisms of dorsoventral polarization (DeRobertis and Sasai, Nature 380, 37 (1996); Arendt and Nübler-Jung, Mech. Develop. 61, 7 (1997)). A prototype molecular strategy for this transformation has emerged from the Drosophila embryo, where the sequential action of a small number of genes results in a ventralizing gradient of the transcription factor Dorsal (St. Johnston and Nüsslein-Volhard, Cell 68, 201 (1992); Morisato and Anderson, Ann. Rev. Genet. 29, 371 (1995)).

This signaling pathway centers on Toll, a transmembrane receptor that transduces the binding of a maternally-secreted ventral factor, Spätzle, into the cytoplasmic engagement of Tube, an accessory molecule, and the activation of Pelle, a Ser/Thr kinase that catalyzes the dissociation of Dorsal from the inhibitor Cactus and allows migration of Dorsal to ventral nuclei (Morisato and Anderson, Ann. Rev. Genet. 29, 371 (1995); Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996)). The Toll pathway also controls the induction of potent antimicrobial factors in the adult fly (Lemaitre, et al., Cell 86, 973 (1996)); this role in Drosophila immune defense strengthens mechanistic parallels to IL-1 pathways that govern a host of immune and inflammatory responses in vertebrates (Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996); Wasserman, Molec. Biol. Cell 4:767 (1993)). A Toll-related cytoplasmic domain in IL-1 receptors directs the binding of a Pelle-like kinase, IRAK, and the activation of a latent NF-κB/I-κB complex that mirrors the embrace of Dorsal and Cactus (Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996); Wasserman, Molec. Biol. Cell 4, 767 (1993)).

We describe the cloning and molecular characterization of four new Toll-like molecules in humans, designated TLRs 2-5 (following Chiang and Beachy, Mech. Develop. 47, 225 (1994)), that reveal a receptor family more closely tied to Drosophila Toll homologs than to vertebrate IL-1 receptors. The TLR sequences are derived from human ESTs; these partial cDNAs were used to draw complete expression profiles in human tissues for the five TLRs, map the chromosomal locations of cognate genes, and narrow the choice of cDNA libraries for full-length cDNA retrievals. Spurred by other efforts (Banfi, et al., Nature Genet. 13, 167 (1996); and Wang, et al., J. Biol. Chem. 271, 4468 (1996)), we are assembling, by structural conservation and molecular parsimony, a biological system in humans that is the counterpart of a compelling regulatory scheme in Drosophila. In addition, a biochemical mechanism driving Toll signaling is suggested by the proposed tertiary fold of the Toll-homology (TH) domain, a core module shared by TLRs, a broad family of IL-1 receptors, mammalian MyD88 factors and plant disease resistance proteins. Mitcham, et al., J. Biol. Chem. 271, 5777 (1996); and Hardiman, et al., Oncogene 13, 2467 (1996). We propose that a signaling route coupling morphogenesis and primitive immunity in insects, plants, and animals (Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996); and Wilson, et al., Curr. Biol. 7, 175 (1997)) may have roots in bacterial two-component pathways.

Toll-like receptor (TLR) molecules belong to the IL-1/Toll receptor family. Ligands for TLR2 and TLR4 have been identified, and their functions are related to the host immune response to microbial antigen or injury. Takeuchi, et al., Immunity 11, 443 (1999); and Noshino, et al., J. Immunol. 162, 3749 (1999). The pattern of expression of TLRs seem to be restricted. Muzio, et al., J. Immunol. 164, 5998 (2000). With these findings that: i) TLR10 is highly expressed and restricted in pDC2s, and ii) pDC2 is the NIPC, it is likely that TLR10 will play an important role in the host's innate immune response.

Computational Analysis.

Human sequences related to insect TLRs were identified from the EST database (dbEST) at the National Center for Biotechnology Information (NCBI) using the BLAST server (Altschul, et al., Nature Genet. 6, 119 (1994)). More sensitive pattern- and profile-based methods (Bork and Gibson, Meth. Enzymol. 266, 162 (1996)) were used to isolate the signaling domains of the TLR family that are shared with vertebrate and plant proteins present in nonredundant databases.

The progressive alignment of TLR intra- or extracellular domain sequences was carried out by ClustalW (Thompson, et al., Nucleic Acids Res. 22, 4673 (1994)); this program also calculated the branching order of aligned sequences by the Neighbor-Joining algorithm (5000 bootstrap replications provided confidence values for the tree groupings).

Conserved alignment patterns, discerned at several degrees of stringency, were drawn by the Consensus program (internet URL http://www.bork.embl-heidelberg .de/Alignment/ consensus. html). The PRINTS library of protein fingerprints(http://www. biochem.ucl. ac.uk/bsm/dbbrowser/ PRINTS/ PRINTS.html) (Attwood, et al., Nucleic Acids Res. 25, 212 (1997)) reliably identified the myriad leucine-rich repeats (LRRs) present in the extracellular segments of TLRs with a compound motif (PRINTS code Leurichrpt) that flexibly matches N- and C-terminal features of divergent LRRs. Two prediction algorithms whose three-state accuracy is above 72% were used to derive a consensus secondary structure for the intracellular domain alignment, as a bridge to fold recognition efforts (Fischer, et al., FASEB J. 10, 126 (1996)). Both the neural network program PHD (Rost and Sander, Proteins 19, 55 (1994)) and the statistical prediction method DSC (King and Sternberg, Protein Sci. 5, 2298 (1996)) have internet servers (URLs http://www. emblheidelberg.de/ predictprotein/phd_pred.html and http://bonsai.lif. icnet.uk/bmm/dsc/dsc_read_align.html, respectively). The intracellular region encodes the THD region discussed, e.g., Hardiman, et al., Oncogene 13, 2467 (1996); Rock, et al., Proc. Nat'l Acad. Sci. USA 95. 588 (1998), each of which is incorporated herein by reference. This domain is very important in the mechanism of signaling by the receptors, which transfers a phosphate group to a substrate.

Cloning of full-length human TLR cDNAs.

PCR primers derived from the Toll-like Humrsc786 sequence (GenBank accession code D13637) (Nomura, et al., DNA Res. 1, 27 (1994)) were used to probe a human erythroleukemic, TF-1 cell line-derived cDNA library (Kitamura, et al., Blood 73, 375 (1989)) to yield the TLR1 cDNA sequence. The remaining TLR sequences were flagged from dbEST, and the relevant EST clones obtained from the I.M.A.G.E. consortium (Lennon, et al., Genomics 33, 151 (1996)) via Research Genetics (Huntsville, AL): CloneID#'s 80633 and 117262 (TLR2), 144675 (TLR3), 202057 (TLR4) and 277229 (TLR5). Full length cDNAs for human TLRs 2-4 were cloned by DNA hybridization screening of λgt10 phage, human adult lung, placenta, and fetal liver 5'-

STRETCH PLUS cDNA libraries (Clontech), respectively; the TLR5 sequence is derived from a human multiple-sclerosis plaque EST. All positive clones were sequenced and aligned to identify individual TLR ORFs: TLR1 (2366 bp clone, 786 aa ORF), TLR2 (2600 bp, 784 aa), TLR3 (3029 bp, 904 aa), TLR4 (3811 bp, 879 aa) and TLR5 (1275 bp, 370 aa). Similar methods are used for TLRs 6-10. Probes for TLR3 and TLR4 hybridizations were generated by PCR using human placenta (Stratagene, La Jolla, CA) and adult liver (Clontech, Palo Alto, CA) cDNA libraries as templates, respectively; primer pairs were derived from the respective EST sequences. PCR reactions were conducted using T. aquaticus TAQPLUS DNA polymerase (Stratagene, La Jolla, CA) under the following conditions: 1 x (94° C, 2 min) 30 x (55° C, 20 sec; 72° C 30 sec; 94° C 20 sec), 1 x (72° C, 8 min). For TLR2 full-length cDNA screening, a 900 bp fragment generated by EcoRI/XbaI digestion of the first EST clone (ID# 80633) was used as a probe.

Northern blots (mRNA) and chromosomal localization.

Human multiple tissue (Cat# 1, 2) and cancer cell line blots (Cat# 7757-1), containing approximately 2 μg of poly(A)$^+$ RNA per lane, were purchased from Clontech (Palo Alto, CA). For TLRs 1-4, the isolated full-length cDNAs served as probes, for TLR5 the EST clone (ID #277229) plasmid insert was used. Briefly, the probes were radiolabeled with [α–^{32}P] dATP using the Amersham REDIPRIME random primer labeling kit (RPN1633). Prehybridization and hybridizations were performed at 65° C in 0.5 M Na_2HPO_4, 7% SDS, 0.5 M EDTA (pH 8.0). All stringency washes were conducted at 65° C with two initial washes in 2 x SSC, 0.1% SDS for 40 min followed by a subsequent wash in 0.1 x SSC, 0.1% SDS for 20 min. Membranes were then exposed at -70° C to X-Ray film (Kodak, Rochester, NY) in the presence of intensifying screens. More detailed studies by cDNA library Southerns (14) were performed with selected human TLR clones to examine their expression in hemopoietic cell subsets.

Human chromosomal mapping was conducted by the method of fluorescence in situ hybridization (FISH) as described in Heng and Tsui, Meth. Molec. Biol. 33, 109 (1994), using the various full-length (TLRs 2-4) or partial (TLR5) cDNA clones as probes. These analyses were performed as a service by SeeDNA Biotech Inc. (Ontario, Canada). A search for human syndromes (or mouse defects in syntenic loci) associated with the mapped TLR genes was conducted in the Dysmorphic Human-Mouse Homology Database by internet server

(http://www.hgmp.mrc.ac.uk/DHMHD/ hum_chrome1.html). Similar methods nare applicable to TLRs 6-10.

Conserved architecture of insect and human TLR ectodomains.

The Toll family in Drosophila comprises at least four distinct gene products: Toll, the prototype receptor involved in dorsoventral patterning of the fly embryo (Morisato and Anderson, Ann. Rev. Genet. 29, 371 (1995)) and a second named '18 Wheeler' (18w) that may also be involved in early embryonic development (Chiang and Beachy, Mech. Develop. 47, 225 (1994); Eldon, et al., Develop. 120, 885 (1994)); two additional receptors are predicted by incomplete, Toll-like ORFs downstream of the male-specific-transcript (Mst) locus (GenBank code X67703) or encoded by the 'sequence-tagged-site' (STS) Dm2245 (GenBank code G01378) (Mitcham, et al., J. Biol. Chem. 271, 5777 (1996)). The extracellular segments of Toll and 18w are distinctively composed of imperfect, ~24 amino acid LRR motifs (Chiang and Beachy, Mech. Develop. 47, 225 (1994); and Eldon, et al., Develop. 120, 885 (1994)). Similar tandem arrays of LRRs commonly form the adhesive antennae of varied cell surface molecules and their generic tertiary structure is presumed to mimic the horseshoe-shaped cradle of a ribonuclease inhibitor fold, where seventeen LRRs show a repeating β/α-hairpin, 28 residue motif (Buchanan and Gay, Prog. Biophys. Molec. Biol. 65, 1 (1996)). The specific recognition of Spätzle by Toll may follow a model proposed for the binding of cystine-knot fold glycoprotein hormones by the multi-LRR ectodomains of serpentine receptors, using the concave side of the curved β-sheet (Kajava, et al., Structure 3, 867 (1995)); intriguingly, the pattern of cysteines in Spätzle, and an orphan Drosophila ligand, Trunk, predict a similar cystine-knot tertiary structure (Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996); and Casanova, et al., Genes Develop. 9, 2539 (1995)).

The 22 and 31 LRR ectodomains of Toll and 18w, respectively (the Mst ORF fragment displays 16 LRRs), are most closely related to the comparable 18, 19, 24, and 22 LRR arrays of TLRs 1-4 (the incomplete TLR5 chain presently includes four membrane-proximal LRRs) by sequence and pattern analysis (Altschul, et al., Nature Genet. 6, 119 (1994); and Bork and Gibson, Meth. Enzymol. 266, 162 (1996)) (Fig. 1). However, a striking difference in the human TLR chains is the common loss of a ~90 residue cysteine-rich region that is variably embedded in the ectodomains of Toll, 18w and the Mst ORF (distanced four, six and two LRRs,

respectively, from the membrane boundary). These cysteine clusters are bipartite, with distinct 'top' (ending an LRR) and 'bottom' (stacked atop an LRR) halves (Chiang and Beachy, Mech. Develop. 47, 225 (1994); Eldon, et al., Develop. 120, 885 (1994); and Buchanan and Gay, Prog. Biophys. Molec. Biol. 65, 1 (1996)); the 'top' module recurs in both Drosophila and human TLRs as a conserved juxtamembrane spacer (Fig. 1). We suggest that the flexibly located cysteine clusters in Drosophila receptors (and other LRR proteins), when mated 'top' to 'bottom', form a compact module with paired termini that can be inserted between any pair of LRRs without altering the overall fold of TLR ectodomains; analogous 'extruded' domains decorate the structures of other proteins (Russell, Protein Engin. 7, 1407 (1994)).

Molecular design of the TH signaling domain.

Sequence comparison of Toll and IL-1 type-I (IL-1R1) receptors has disclosed a distant resemblance of a ~200 amino acid cytoplasmic domain that presumably mediates signaling by similar Rel-type transcription factors (Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996); Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996); Wasserman, Molec. Biol. Cell 4, 767 (1993)). More recent additions to this functional paradigm include a pair of plant disease resistance proteins from tobacco and flax that feature an N-terminal TH module followed by nucleotide-binding (NTPase) and LRR segments (Wilson, et al., Curr. Biol. 7, 175 (1997)); by contrast, a 'death domain' precedes the TH chain of MyD88, an intracellular myeloid differentiation marker (Mitcham, et al., J. Biol. Chem. 271, 5777 (1996); and Hardiman, et al., Oncogene 13, 2467 (1996)) (Fig. 1). New IL-1-type receptors include IL-1R3, an accessory signaling molecule, and orphan receptors IL-1R4 (also called ST2/Fit-1/T1), IL-1R5 (IL-1R-related protein), and IL-1R6 (IL-1R-related protein-2) (Mitcham, et al., J. Biol. Chem. 271:5777 (1996);Hardiman, et al., Oncogene 13, 2467 (1996)). With the new human TLR sequences, we have sought a structural definition of this evolutionary thread by analyzing the conformation of the common TH module: ten blocks of conserved sequence comprising 128 amino acids form the minimal TH domain fold; gaps in the alignment mark the likely location of sequence and length-variable loops (Fig. 2A-2B).

Two prediction algorithms that take advantage of the patterns of conservation and variation in multiply aligned sequences, PHD (Rost and Sander, Proteins 19, 55 (1994)) and DSC (King and Sternberg, Protein Sci. 5, 2298 (1996)), produced strong, concordant results for

the TH signaling module (Fig. 2A-2B). Each block contains a discrete secondary structural element: the imprint of alternating β-strands (labeled A-E) and α-helices (numbered 1-5) is diagnostic of a β/α-class fold with α-helices on both faces of a parallel β-sheet. Hydrophobic β-strands A, C and D are predicted to form 'interior' staves in the β-sheet, while the shorter, amphipathic β-strands B and E resemble typical 'edge' units (Fig. 2A-2B). This assignment is consistent with a strand order of B-A-C-D-E in the core β-sheet (Fig. 2C); fold comparison ('mapping') and recognition ('threading') programs (Fischer, et al., FASEB J. 10, 126 (1996)) strongly return this doubly wound β/α topology. A surprising, functional prediction of this outline structure for the TH domain is that many of the conserved, charged residues in the multiple alignment map to the C-terminal end of the β-sheet: residue Asp16 (block numbering scheme - Fig. 2A-2B) at the end of βA, Arg39 and Asp40 following βB, Glu75 in the first turn of α3, and the more loosely conserved Glu/Asp residues in the βD-α4 loop, or after βE (Fig. 2A-2B). The location of four other conserved residues (Asp7, Glu28, and the Arg57-Arg/Lys58 pair) is compatible with a salt bridge network at the opposite, N-terminal end of the β-sheet (Fig. 2A-2B). Alignment of the other TLR embodiments exhibit similar features, and peptide segments comprising these feataures, e.g., 20 amino acid segments containing them, are particularly important.

Signaling function depends on the structural integrity of the TH domain. Inactivating mutations or deletions within the module boundaries (Fig. 2A-2B) have been catalogued for IL-1R1 and Toll (Heguy, et al., J. Biol. Chem. 267, 2605 (1992); Croston, et al., J. Biol. Chem. 270, 16514 (1995); Schneider, et al., Genes Develop. 5, 797 (1991); Norris and Manley, Genes Develop. 6, 1654 (1992); Norris and Manley, Genes Develop. 9, 358 (1995); Norris and Manley, Genes Develop. 10, 862 (1996)). The human TLR1-5 chains extending past the minimal TH domain (8, 0, 6, 22 and 18 residue lengths, respectively) are most closely similar to the stubby, 4 aa 'tail' of the Mst ORF. Toll and 18w display unrelated 102 and 207 residue tails (Fig. 2A-2B) that may negatively regulate the signaling of the fused TH domains (Norris and Manley, Genes Develop. 9, 358 (1995); Norris and Manley, Genes Develop. 10, 862 (1996)).

The evolutionary relationship between the disparate proteins that carry the TH domain can best be discerned by a phylogenetic tree derived from the multiple alignment (Fig. 3). Four principal branches segregate the plant proteins, the MyD88 factors, IL-1 receptors, and Toll-like molecules; the latter branch clusters the Drosophila and human TLRs.

Chromosomal dispersal of human TLR genes.

In order to investigate the genetic linkage of the nascent human TLR gene family, we mapped the chromosomal loci of four of the five genes by FISH (Fig. 4). The TLR1 gene has previously been charted by the human genome project: an STS database locus (dbSTS accession number G06709, corresponding to STS WI-7804 or SHGC-12827) exists for the Humrsc786 cDNA (Nomura, et al., DNA Res. 1, 27 (1994)) and fixes the gene to chromosome 4 marker interval D4S1587-D42405 (50-56 cM) circa 4p14. This assignment has recently been corroborated by FISH analysis. Taguchi, et al., Genomics 32, 486 (1996). In the present work, we reliably assign the remaining TLR genes to loci on chromosome 4q32 (TLR2), 4q35 (TLR3), 9q32-33 (TLR4) and 1q33.3 (TLR5). During the course of this work, an STS for the parent TLR2 EST (cloneID # 80633) has been generated (dbSTS accession number T57791 for STS SHGC-33147) and maps to the chromosome 4 marker interval D4S424-D4S1548 (143-153 cM) at 4q32 -in accord with our findings. There is a ~50 cM gap between TLR2 and TLR3 genes on the long arm of chromosome 4.

TLR genes are differentially expressed.

Both Toll and 18w have complex spatial and temporal patterns of expression in Drosophila that may point to functions beyond embryonic patterning (St. Johnston and Nüsslein-Volhard, Cell 68, 201 (1992); Morisato and Anderson, Ann. Rev. Genet. 29, 371 (1995); Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996); Lemaitre, et al., Cell 86, 973 (1996); Chiang and Beachy, Mech. Develop. 47, 225 (1994); Eldon, et al., Develop. 120, 885 (1994)). We have examined the spatial distribution of TLR transcripts by mRNA blot analysis with varied human tissue and cancer cell lines using radiolabeled TLR cDNAs (Fig. 5). TLR1 is found to be ubiquitously expressed, and at higher levels than the other receptors. Presumably reflecting alternative splicing, 'short' 3.0 kB and 'long' 8.0 kB TLR1 transcript forms are present in ovary and spleen, respectively (Fig. 5, panels A and B). A cancer cell mRNA panel also shows the prominent overexpression of TLR1 in a Burkitt's Lymphoma Raji cell line (Fig. 5, panel C). TLR2 mRNA is less widely expressed than TLR1, with a 4.0 kB species detected in lung and a 4.4 kB transcript evident in heart, brain and muscle. The tissue distribution pattern of TLR3 echoes that of TLR2 (Fig. 5, panel E). TLR3 is also present as two major transcripts of

approximately 4.0 and 6.0 kB in size, and the highest levels of expression are observed in placenta and pancreas. By contrast, TLR4 and TLR5 messages appear to be extremely tissue-specific. TLR4 was detected only in placenta as a single transcript of ~7.0 kB in size. A faint 4.0 kB signal was observed for TLR5 in ovary and peripheral blood monocytes.

Components of an evolutionarily ancient regulatory system.

The original molecular blueprints and divergent fates of signaling pathways can be reconstructed by comparative genomic approaches (Miklos and Rubin, Cell 86, 521 (1996); Chothia, Develop. 1994 Suppl., 27 (1994); Banfi, et al., Nature Genet. 13, 167 (1996); Wang, et al., J. Biol. Chem. 271, 4468 (1996)). We have used this logic to identify an emergent gene family in humans, encoding five receptor paralogs at present, TLRs 1-5, that are the direct evolutionary counterparts of a Drosophila gene family headed by Toll (Figs. 1-3). The conserved architecture of human and fly TLRs, conserved LRR ectodomains and intracellular TH modules (Fig. 1), intimates that the robust pathway coupled to Toll in Drosophila (6, 7) survives in vertebrates. The best evidence borrows from a reiterated pathway: the manifold IL-1 system and its repertoire of receptor-fused TH domains, IRAK, NF-κB and I-κB homologs (Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996); Wasserman, Molec. Biol. Cell 4, 767 (1993); Hardiman, et al., Oncogene 13, 2467 (1996); Cao, et al., Science 271, 1128 (1996)); a Tube-like factor has also been characterized. It is not known whether TLRs can productively couple to the IL-1R signaling machinery, or instead, a parallel set of proteins is used. Differently from IL-1 receptors, the LRR cradle of human TLRs is predicted to retain an affinity for Spätzle/Trunk-related cystine-knot factors; candidate TLR ligands (called PENs) that fit this mold have been isolated.

Biochemical mechanisms of signal transduction can be gauged by the conservation of interacting protein folds in a pathway (Miklos and Rubin, Cell 86, 521 (1996); Chothia, Develop. 1994 Suppl., 27 (1994)). At present, the Toll signaling paradigm involves some molecules whose roles are narrowly defined by their structures, actions or fates: Pelle is a Ser/Thr kinase (phosphorylation), Dorsal is an NF-κB-like transcription factor (DNA-binding) and Cactus is an ankyrin-repeat inhibitor (Dorsal binding, degradation) (Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996)). By contrast, the functions of the Toll TH domain and Tube remain enigmatic. Like other cytokine receptors (Heldin, Cell 80, 213 (1995)), ligand-mediated

dimerization of Toll appears to be the triggering event: free cysteines in the juxtamembrane region of Toll create constitutively active receptor pairs (Schneider, et al., Genes Develop. 5, 797 (1991)), and chimeric Torso-Toll receptors signal as dimers (Galindo, et al., Develop. 121, 2209 (1995)); yet, severe truncations or wholesale loss of the Toll ectodomain results in promiscuous intracellular signaling (Norris and Manley, Genes Develop. 9, 358 (1995); Winans and Hashimoto, Molec. Biol. Cell 6, 587 (1995)), reminiscent of oncogenic receptors with catalytic domains (Heldin, Cell 80, 213 (1995)). Tube is membrane-localized, engages the N-terminal (death) domain of Pelle and is phosphorylated, but neither Toll-Tube or Toll-Pelle interactions are registered by two-hybrid analysis (Galindo, et al., Develop. 121, 2209 (1995); Groβhans, et al., Nature 372, 563 (1994)); this latter result suggests that the conformational 'state' of the Toll TH domain somehow affects factor recruitment (Norris and Manley, Genes Develop. 10, 862 (1996); and Galindo, et al., Develop. 121, 2209 (1995)).

At the heart of these vexing issues is the structural nature of the Toll TH module. To address this question, we have taken advantage of the evolutionary diversity of TH sequences from insects, plants and vertebrates, incorporating the human TLR chains, and extracted the minimal, conserved protein core for structure prediction and fold recognition (Fig. 2). The strongly predicted $(\beta/\alpha)_5$ TH domain fold with its asymmetric cluster of acidic residues is topologically identical to the structures of response regulators in bacterial two-component signaling pathways (Volz, Biochemistry 32, 11741 (1993); and Parkinson, Cell 73, 857 (1993)) (Fig. 2A-2C). The prototype chemotaxis regulator CheY transiently binds a divalent cation in an 'aspartate pocket' at the C-end of the core β-sheet; this cation provides electrostatic stability and facilitates the activating phosphorylation of an invariant Asp (Volz, Biochemistry 32, 11741 (1993)). Likewise, the TH domain may capture cations in its acidic nest, but activation, and downstream signaling, could depend on the specific binding of a negatively charged moiety: anionic ligands can overcome intensely negative binding-site potentials by locking into precise hydrogen-bond networks (Ledvina, et al., Proc. Natl. Acad. Sci. USA 93, 6786 (1996)). Intriguingly, the TH domain may not simply act as a passive scaffold for the assembly of a Tube/Pelle complex for Toll, or homologous systems in plants and vertebrates, but instead actively participate as a true conformational trigger in the signal transducing machinery. Perhaps explaining the conditional binding of a Tube/Pelle complex, Toll dimerization could promote unmasking, by regulatory receptor tails (Norris and Manley, Genes Develop. 9, 358 (1995);

Norris and Manley, Genes Develop. 10, 862 (1996)), or binding by small molecule activators of the TH pocket. However, 'free' TH modules inside the cell (Norris and Manley, Genes Develop. 9, 358 (1995); Winans and Hashimoto, Molec. Biol. Cell 6, 587 (1995)) could act as catalytic, CheY-like triggers by activating and docking with errant Tube/Pelle complexes.

Morphogenetic receptors and immune defense.

The evolutionary link between insect and vertebrate immune systems is stamped in DNA: genes encoding antimicrobial factors in insects display upstream motifs similar to acute phase response elements known to bind NF-κB transcription factors in mammals (Hultmark, Trends Genet. 9, 178 (1993)). Dorsal, and two Dorsal-related factors, Dif and Relish, help induce these defense proteins after bacterial challenge (Reichhart, et al., C. R. Acad. Sci. Paris 316, 1218 (1993); Ip, et al., Cell 75, 753 (1993); Dushay, et al., Proc. Natl. Acad. Sci. USA 93, 10343 (1996)); Toll, or other TLRs, likely modulate these rapid immune responses in adult Drosophila (Lemaitre, et al. (1996) Cell 86:973-983; Rosetto, et al., Biochem. Biophys. Res. Commun. 209, 111 (1995)). These mechanistic parallels to the IL-1 inflammatory response in vertebrates are evidence of the functional versatility of the Toll signaling pathway, and suggest an ancient synergy between embryonic patterning and innate immunity (Belvin and Anderson, Ann. Rev. Cell Develop. Biol. 12, 393 (1996); Lemaitre, et al., Cell 86, 973 (1996); Wasserman, Molec. Biol. Cell 4, 767 (1993); Wilson, et al., Curr. Biol. 7, 175 (1997); Hultmark, Trends Genet. 9, 178 (1993); Reichhart, et al., C. R. Acad. Sci. Paris 316, 1218 (1993); Ip, et al., Cell 75, 753 (1993); Dushay, et al., Proc. Natl. Acad. Sci. USA 93, 10343 (1996); Rosetto, et al., Biochem. Biophys. Res. Commun. 209, 111 (1995); Medzhitov and Janeway, Curr. Opin. Immunol. 9, 4 (1997)). The closer homology of insect and human TLR proteins invites an even stronger overlap of biological functions that supersedes the purely immune parallels to IL-1 systems, and lends potential molecular regulators to dorso-ventral and other transformations of vertebrate embryos (DeRobertis and Sasai, Nature 380, 37 (1996); Arendt and Nübler-Jung, Mech. Develop. 61, 7 (1997)).

The present description of an emergent, robust receptor family in humans mirrors the recent discovery of the vertebrate Frizzled receptors for Wnt patterning factors. Wang, et al., J. Biol. Chem. 271, 4468 (1996). As numerous other cytokine-receptor systems have roles in early development (Lemaire and Kodjabachian, Trends Genet. 12, 525 (1996)), perhaps the distinct

cellular contexts of compact embryos and gangly adults simply result in familiar signaling pathways and their diffusible triggers having different biological outcomes at different times, e.g., morphogenesis versus immune defense for TLRs. For insect, plant, and human Toll-related systems (Hardiman, et al., Oncogene 13, 2467 (1996); Wilson, et al., Curr. Biol. 7, 175 (1997), these signals course through a regulatory TH domain that intriguingly resembles a bacterial transducing engine (Parkinson, Cell 73, 857 (1993)).

In particular, the TLR6 exhibits structural features which establish its membership in the family. Moreover, members of the family have been implicated in a number of significant developmental disease conditions and with function of the innate immune system. In particular, the TLR6 has been mapped to the X chromosome to a location which is a hot spot for major developmental abnormalities. See, e.g., The Sanger Center: human X chromosome website http://www.sanger.ac.uk/HGP/ChrX/index.shtml; and the Baylor College of Medicine Human Genome Sequencing website http://gc.bcm.tmc.edu:8088/cgi-bin/seq/home.

The accession number for the deposited PAC is AC003046. This accession number contains sequence from two PACs: RPC-164K3 and RPC-263P4. These two PAC sequences mapped on human chromosome Xp22 at the Baylor web site between STS markers DXS704 and DXS7166. This region is a "hot spot" for severe developmental abnormalities.

EXAMPLE III. Amplification of TLR fragment by PCR

Two appropriate primer sequences are selected (see Tables 1 through 10). RT-PCR is used on an appropriate mRNA sample selected for the presence of message to produce a partial or full length cDNA, e.g., a sample which expresses the gene. See, e.g., Innis, et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, CA (1990); and Dieffenbach and Dveksler, PCR Primer: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1995). Such will allow determination of a useful sequence to probe for a full length gene in a cDNA library. The TLR6 is a contiguous sequence in the genome, which may suggest that the other TLRs are also. Thus, PCR on genomic DNA may yield full length contiguous sequence, and chromosome walking methodology would then be applicable. Alternatively, sequence databases will contain sequence corresponding to portions of the described embodiments, or closely related forms, e.g., alternative splicing, etc. Expression cloning techniques also may be applied on cDNA libraries.

EXAMPLE IV. Tissue distribution of TLRs

Message for each gene encoding these TLRs has been detected. See Figures 5A-5F. Other cells and tissues will be assayed by appropriate technology, e.g., PCR, immunoassay, hybridization, or otherwise. Tissue and organ cDNA preparations are available, e.g., from Clontech, Mountain View, CA. Identification of sources of natural expression are useful, as described.

Southern Analysis: DNA (5 μg) from a primary amplified cDNA library is digested with appropriate restriction enzymes to release the inserts, run on a 1% agarose gel and transferred to a nylon membrane (Schleicher and Schuell, Keene, NH).

Samples for human mRNA isolation would typically include, e.g.: peripheral blood mononuclear cells (monocytes, T cells, NK cells, granulocytes, B cells), resting (T100); peripheral blood mononuclear cells, activated with anti-CD3 for 2, 6, 12 h pooled (T101); T cell, TH0 clone Mot 72, resting (T102); T cell, TH0 clone Mot 72, activated with anti-CD28 and anti-CD3 for 3, 6, 12 h pooled (T103); T cell, TH0 clone Mot 72, anergic treated with specific peptide for 2, 7, 12 h pooled (T104); T cell, TH1 clone HY06, resting (T107); T cell, TH1 clone HY06, activated with anti-CD28 and anti-CD3 for 3, 6, 12 h pooled (T108); T cell, TH1 clone HY06, anergic treated with specific peptide for 2, 6, 12 h pooled (T109); T cell, TH2 clone HY935, resting (T110); T cell, TH2 clone HY935, activated with anti-CD28 and anti-CD3 for 2, 7, 12 h pooled (T111); T cells CD4+CD45RO- T cells polarized 27 days in anti-CD28, IL-4, and anti IFN-γ, TH2 polarized, activated with anti-CD3 and anti-CD28 4 h (T116); T cell tumor lines Jurkat and Hut78, resting (T117); T cell clones, pooled AD130.2, Tc783.12, Tc783.13, Tc783.58, Tc782.69, resting (T118); T cell random γδ T cell clones, resting (T119); Splenocytes, resting (B100); Splenocytes, activated with anti-CD40 and IL-4 (B101); B cell EBV lines pooled WT49, RSB, JY, CVIR, 721.221, RM3, HSY, resting (B102); B cell line JY, activated with PMA and ionomycin for 1, 6 h pooled (B103); NK 20 clones pooled, resting (K100); NK 20 clones pooled, activated with PMA and ionomycin for 6 h (K101); NKL clone, derived from peripheral blood of LGL leukemia patient, IL-2 treated (K106); NK cytotoxic clone 640-A30-1, resting (K107); hematopoietic precursor line TF1, activated with PMA and ionomycin for 1, 6 h pooled (C100); U937 premonocytic line, resting (M100); U937 premonocytic line, activated with PMA and ionomycin for 1, 6 h pooled (M101); elutriated monocytes, activated with LPS, IFNγ,

anti-IL-10 for 1, 2, 6, 12, 24 h pooled (M102); elutriated monocytes, activated with LPS, IFNγ, IL-10 for 1, 2, 6, 12, 24 h pooled (M103); elutriated monocytes, activated with LPS, IFNγ, anti-IL-10 for 4, 16 h pooled (M106); elutriated monocytes, activated with LPS, IFNγ, IL-10 for 4, 16 h pooled (M107); elutriated monocytes, activated LPS for 1 h (M108); elutriated monocytes, activated LPS for 6 h (M109); DC 70% CD1a+, from CD34+ GM-CSF, TNFα 12 days, resting (D101); DC 70% CD1a+, from CD34+ GM-CSF, TNFα 12 days, activated with PMA and ionomycin for 1 hr (D102); DC 70% CD1a+, from CD34+ GM-CSF, TNFα 12 days, activated with PMA and ionomycin for 6 hr (D103); DC 95% CD1a+, from CD34+ GM-CSF, TNFα 12 days FACS sorted, activated with PMA and ionomycin for 1, 6 h pooled (D104); DC 95% CD14+, ex CD34+ GM-CSF, TNFα 12 days FACS sorted, activated with PMA and ionomycin 1, 6 hr pooled (D105); DC CD1a+ CD86+, from CD34+ GM-CSF, TNFα 12 days FACS sorted, activated with PMA and ionomycin for 1, 6 h pooled (D106); DC from monocytes GM-CSF, IL-4 5 days, resting (D107); DC from monocytes GM-CSF, IL-4 5 days, resting (D108); DC from monocytes GM-CSF, IL-4 5 days, activated LPS 4, 16 h pooled (D109); DC from monocytes GM-CSF, IL-4 5 days, activated TNFα, monocyte supe for 4, 16 h pooled (D110); leiomyoma L11 benign tumor (X101); normal myometrium M5 (O115); malignant leiomyosarcoma GS1 (X103); lung fibroblast sarcoma line MRC5, activated with PMA and ionomycin for 1, 6 h pooled (C101); kidney epithelial carcinoma cell line CHA, activated with PMA and ionomycin for 1, 6 h pooled (C102); kidney fetal 28 wk male (O100); lung fetal 28 wk male (O101); liver fetal 28 wk male (O102); heart fetal 28 wk male (O103); brain fetal 28 wk male (O104); gallbladder fetal 28 wk male (O106); small intestine fetal 28 wk male (O107); adipose tissue fetal 28 wk male (O108); ovary fetal 25 wk female (O109); uterus fetal 25 wk female (O110); testes fetal 28 wk male (O111); spleen fetal 28 wk male (O112); adult placenta 28 wk (O113); and tonsil inflamed, from 12 year old (X100).

Samples for mouse mRNA isolation can include, e.g.: resting mouse fibroblastic L cell line (C200); Braf:ER (Braf fusion to estrogen receptor) transfected cells, control (C201); T cells, TH1 polarized (Mel14 bright, CD4+ cells from spleen, polarized for 7 days with IFN-γ and anti IL-4; T200); T cells, TH2 polarized (Mel14 bright, CD4+ cells from spleen, polarized for 7 days with IL-4 and anti-IFN-γ; T201); T cells, highly TH1 polarized (see Openshaw, et al., J. Exp. Med. 182, 1357 (1995); activated with anti-CD3 for 2, 6, 16 h pooled; T202); T cells, highly TH2 polarized (Openshaw, et al., J. Exp. Med. 182, 1357 (1995)); activated with anti-CD3 for 2,

6, 16 h pooled; T203); CD44- CD25+ pre T cells, sorted from thymus (T204); TH1 T cell clone D1.1, resting for 3 weeks after last stimulation with antigen (T205); TH1 T cell clone D1.1, 10 μg/ml ConA stimulated 15 h (T206); TH2 T cell clone CDC35, resting for 3 weeks after last stimulation with antigen (T207); TH2 T cell clone CDC35, 10 μg/ml ConA stimulated 15 h (T208); Mel14+ naive T cells from spleen, resting (T209); Mel14+ T cells, polarized to Th1 with IFN-γ/IL-12/anti-IL-4 for 6, 12, 24 h pooled (T210); Mel14+ T cells, polarized to Th2 with IL-4/anti-IFN-γ for 6, 13, 24 h pooled (T211); unstimulated mature B cell leukemia cell line A20 (B200); unstimulated B cell line CH12 (B201); unstimulated large B cells from spleen (B202); B cells from total spleen, LPS activated (B203); metrizamide enriched dendritic cells from spleen, resting (D200); dendritic cells from bone marrow, resting (D201); monocyte cell line RAW 264.7 activated with LPS 4 h (M200); bone-marrow macrophages derived with GM and M-CSF (M201); macrophage cell line J774, resting (M202); macrophage cell line J774 + LPS + anti-IL-10 at 0.5, 1, 3, 6, 12 h pooled (M203); macrophage cell line J774 + LPS + IL-10 at 0.5, 1, 3, 5, 12 h pooled(M204); aerosol challenged mouse lung tissue, Th2 primers, aerosol OVA challenge 7, 14, 23 h pooled (see Garlisi, et al., Clinical Immunology and Immunopathology 75, 75(1995); X206); Nippostrongulus-infected lung tissue (see Coffman, et al., Science 245, 308 (1989); X200); total adult lung, normal (O200); total lung, rag-1 (Schwarz, et al., Immunodeficiency 4, 249 (1993)); O205); IL-10 K.O. spleen (see Kuhn, et al., Cell 75, 263 (1991); X201); total adult spleen, normal (O201); total spleen, rag-1 (O207); IL-10 K.O. Peyer's patches (O202); total Peyer's patches, normal (O210); IL-10 K.O. mesenteric lymph nodes (X203); total mesenteric lymph nodes, normal (O211); IL-10 K.O. colon (X203); total colon, normal (O212); NOD mouse pancreas (see Makino, et al., Jikken Dobutsu 29, 1 (1980); X205); total thymus, rag-1 (O208); total kidney, rag-1 (O209); total heart, rag-1 (O202); total brain, rag-1 (O203); total testes, rag-1 (O204); total liver, rag-1 (O206); rat normal joint tissue (O300); and rat arthritic joint tissue (X300).

The TLR10 has been found to be highly expressed in precursor dendritic cell type 2 (pDC2). See, e.g., Rissoan, et al., Science 283, 1183 (1999); and Siegal, et al., Science 284, 1835 (1999). However, it is not expressed on monocytes. The restricted expression of TLR10 reinforces the suggestions of a role for the receptor in host immune defense. The pDC2 cells are natural interferon producing cells (NIPC), which produce large amounts of IFNα in response to Herpes simplex virus infection.

EXAMPLE V. Cloning of species counterparts of TLRs

Various strategies are used to obtain species counterparts of these TLRs, preferably from other primates. One method is by cross hybridization using closely related species DNA probes. It may be useful to go into evolutionarily similar species as intermediate steps. Another method is by using specific PCR primers based on the identification of blocks of similarity or difference between particular species, e.g., human, genes, e.g., areas of highly conserved or nonconserved polypeptide or nucleotide sequence. Alternatively, antibodies may be used for expression cloning.

EXAMPLE VI. Production of mammalian TLR protein

An appropriate, e.g., GST, fusion construct is engineered for expression, e.g., in E. coli. For example, a mouse IGIF pGex plasmid is constructed and transformed into E. coli. Freshly transformed cells are grown in LB medium containing 50 μg/ml ampicillin and induced with IPTG (Sigma, St. Louis, MO). After overnight induction, the bacteria are harvested and the pellets containing the TLR protein are isolated. The pellets are homogenized in TE buffer (50 mM Tris-base pH 8.0, 10 mM EDTA and 2 mM pefabloc) in 2 liters. This material is passed through a microfluidizer (Microfluidics, Newton, MA) three times. The fluidized supernatant is centrifuged in a Sorvall GS-3 rotor for 1 h at 13,000 rpm. The resulting supernatant containing the TLR protein is filtered and passed over a glutathione-SEPHAROSE column equilibrated in 50 mM Tris-base pH 8.0. The fractions containing the TLR-GST fusion protein are pooled and cleaved with thrombin (Enzyme Research Laboratories, Inc., South Bend, IN). The cleaved pool is then passed over a Q-SEPHAROSE column equilibrated in 50 mM Tris-base. Fractions containing TLR are pooled and diluted in cold distilled H_2O, to lower the conductivity, and passed back over a fresh Q-Sepharose column, alone or in succession with an immunoaffinity antibody column.. Fractions containing the TLR protein are pooled, aliquoted, and stored in the -70° C freezer.

Comparison of the CD spectrum with TLR1 protein may suggest that the protein is correctly folded (Hazuda, et al., J. Biol. Chem. 264, 1689 (1969)).

EXAMPLE VII. Biological Assays with TLRs

Biological assays will generally be directed to the ligand binding feature of the protein or to the kinase/phosphatase activity of the receptor. The activity will typically be reversible, as are many other enzyme actions, and will mediate phosphatase or phosphorylase activities, which activities are easily measured by standard procedures. See, e.g., Hardie, et al., The Protein Kinase FactBook vols. I and II, Academic Press, San Diego (1995), CA; Hanks, et al., Meth. Enzymol. 200, 38 (1991); Hunter, et al., Cell 70, 375 (1992); Lewin, Cell 61, 743-752 (1990); Pines, et al. (1991) Cold Spring Harbor Symp. Quant. Biol. 56, 449 (1991); and Parker, et al., Nature 363, 736 (1993). Because of the homology of the cytoplasmic domain of the Toll receptor and the cytoplasmic domain of the IL-1 receptor, assays sensitive to IL-1 receptor activity may be suitable for measuring activity of TLRs. A review of IL-1 receptor mediated activities is available (Dinarello, Blood 87, 2095 (1996)).

EXAMPLE VIII. Preparation of antibodies specific for TLR, e.g., TLR4

Inbred Balb/c mice are immunized intraperitoneally with recombinant forms of the protein, e.g., purified TLR4 or stable transfected NIH-3T3 cells. Animals are boosted at appropriate time points with protein, with or without additional adjuvant, to further stimulate antibody production. Serum is collected, or hybridomas produced with harvested spleens.

Alternatively, Balb/c mice are immunized with cells transformed with the gene or fragments thereof, either endogenous or exogenous cells, or with isolated membranes enriched for expression of the antigen. Serum is collected at the appropriate time, typically after numerous further administrations. Various gene therapy techniques may be useful, e.g., in producing protein in situ, for generating an immune response.

Monoclonal antibodies may be made. For example, splenocytes are fused with an appropriate fusion partner and hybridomas are selected in growth medium by standard procedures. Hybridoma supernatants are screened for the presence of antibodies which bind to the desired TLR, e.g., by ELISA or other assay. Antibodies which specifically recognize specific TLR embodiments may also be selected or prepared.

In another method, synthetic peptides or purified protein are presented to an immune system to generate monoclonal or polyclonal antibodies. See, e.g., Coligan, Current Protocols in Immunology Wiley/Greene (1991); and Harlow and Lane, Antibodies: A Laboratory Manual

Cold Spring Harbor Press (1989). In appropriate situations, the binding reagent is either labeled as described above, e.g., fluorescence or otherwise, or immobilized to a substrate for panning methods. Nucleic acids may also be introduced into cells in an animal to produce the antigen, which serves to elicit an immune response. See, e.g., Wang, et al., Proc. Nat'l. Acad. Sci. 90, 4156 (1993); Barry, et al., BioTechniques 16, 616 (1994); and Xiang, et al., Immunity 2, 129 (1995).

EXAMPLE IX. Production of fusion proteins with TLR, e.g., TLR5

Various fusion constructs are made with TLR5. This portion of the gene is fused to an epitope tag, e.g., a FLAG tag, or to a two hybrid system construct. See, e.g., Fields and Song, Nature 340, 245 (1989).

The epitope tag may be used in an expression cloning procedure with detection with anti-FLAG antibodies to detect a binding partner, e.g., ligand for the respective TLR5. The two hybrid system may also be used to isolate proteins which specifically bind to TLR5.

EXAMPLE X. Chromosomal mapping of TLRs

Chromosome spreads are prepared. In situ hybridization is performed on chromosome preparations obtained from phytohemagglutinin-stimulated lymphocytes cultured for 72 h. 5-bromodeoxyuridine is added for the final seven hours of culture (60 μg/ml of medium), to ensure a posthybridization chromosomal banding of good quality.

An appropriate fragment, e.g., a PCR fragment, amplified with the help of primers on total B cell cDNA template, is cloned into an appropriate vector. The vector is labeled by nick-translation with ^{3}H. The radiolabeled probe is hybridized to metaphase spreads as described by Mattei, et al., Hum. Genet. 69, 327 (1985).

After coating with nuclear track emulsion (KODAK NTB_2), slides are exposed, e.g., for 18 days at 4° C. To avoid any slipping of silver grains during the banding procedure, chromosome spreads are first stained with buffered Giemsa solution and metaphase photographed.

R-banding is then performed by the fluorochrome-photolysis-Giemsa (FPG) method and metaphases rephotographed before analysis.

Alternatively, FISH can be performed, as described above. The TLR genes are located on different chromosomes. TLR2 and TLR3 are localized to human chromosome 4; TLR4 is localized to human chromosome 9, and TLR5 is localized to human chromosome 1. See Figures 4A-4D.

EXAMPLE XI. Isolation of a ligand for a TLR

A TLR can be used as a specific binding reagent to identify its binding partner, by taking advantage of its specificity of binding, much like an antibody would be used. A binding reagent is either labeled as described above, e.g., fluorescence or otherwise, or immobilized to a substrate for panning methods.

The binding composition is used to screen an expression library made from a cell line which expresses a binding partner, i.e., ligand, preferably membrane associated. Standard staining techniques are used to detect or sort surface expressed ligand, or surface expressing transformed cells are screened by panning. Screening of intracellular expression is performed by various staining or immunofluorescence procedures. See also McMahan, et al., EMBO J. 10, 2821 (1991).

For example, on day 0, precoat 2-chamber permanox slides with 1 ml per chamber of fibronectin, 10 ng/ml in PBS, for 30 min at room temperature. Rinse once with PBS. Then plate COS cells at 2-3 x 10^5 cells per chamber in 1.5 ml of growth media. Incubate overnight at 37° C.

On day 1 for each sample, prepare 0.5 ml of a solution of 66 µg/ml DEAE-dextran, 66 µM chloroquine, and 4 µg DNA in serum free DME. For each set, a positive control is prepared, e.g., of TLR-FLAG cDNA at 1 and 1/200 dilution, and a negative mock. Rinse cells with serum free DME. Add the DNA solution and incubate 5 hr at 37° C. Remove the medium and add 0.5 ml 10% DMSO in DME for 2.5 min. Remove and wash once with DME. Add 1.5 ml growth medium and incubate overnight.

On day 2, change the medium. On days 3 or 4, the cells are fixed and stained. Rinse the cells twice with Hank's Buffered Saline Solution (HBSS) and fix in 4% paraformaldehyde/glucose for 5 min. Wash 3X with HBSS. The slides may be stored at -80° C after all liquid is removed. For each chamber, 0.5 ml incubations are performed as follows. Add HBSS/saponin (0.1%) with 32 µl/ml of 1 M NaN_3 for 20 min. Cells are then washed with

HBSS/saponin 1X. Add appropriate TLR or TLR/antibody complex to cells and incubate for 30 min. Wash cells twice with HBSS/saponin. If appropriate, add first antibody for 30 min. Add second antibody, e.g., Vector anti-mouse antibody, at 1/200 dilution, and incubate for 30 min. Prepare ELISA solution, e.g., Vector Elite ABC horseradish peroxidase solution, and preincubate for 30 min. Use, e.g., 1 drop of solution A (avidin) and 1 drop solution B (biotin) per 2.5 ml HBSS/saponin. Wash cells twice with HBSS/saponin. Add ABC HRP solution and incubate for 30 min. Wash cells twice with HBSS, second wash for 2 min, which closes cells. Then add Vector diaminobenzoic acid (DAB) for 5 to 10 min. Use 2 drops of buffer plus 4 drops DAB plus 2 drops of H_2O_2 per 5 ml of glass distilled water. Carefully remove chamber and rinse slide in water. Air dry for a few minutes, then add 1 drop of Crystal Mount and a cover slip. Bake for 5 min at 85-90° C.

Evaluate positive staining of pools and progressively subclone to isolation of single genes responsible for the binding.

Alternatively, TLR reagents are used to affinity purify or sort out cells expressing a putative ligand. See, e.g., Sambrook, et al. or Ausubel, et al.

Another strategy is to screen for a membrane bound receptor by panning. The receptor cDNA is constructed as described above. The ligand can be immobilized and used to immobilize expressing cells. Immobilization may be achieved by use of appropriate antibodies which recognize, e.g., a FLAG sequence of a TLR fusion construct, or by use of antibodies raised against the first antibodies. Recursive cycles of selection and amplification lead to enrichment of appropriate clones and eventual isolation of receptor expressing clones.

Phage expression libraries can be screened by mammalian TLRs. Appropriate label techniques, e.g., anti-FLAG antibodies, will allow specific labeling of appropriate clones.

EXAMPLE XII. Differentiation of pre-dendritic cells to mature myeloid cells and differentiation of naive T helper cells to T_H1 cells; differentiation of pre-dendritic cells to mature lymphoid-type cells and differentiation of naive T helper cells to T_H2 cells.

Dendritic cells participate in the *innate immune system*, as these cells contain Toll-like receptors which can respond to molecules specific to bacteria, such as bacterial lipopolysacchardise (endotoxin), lipoteichoic acid, and non-methylated CpG oligonucleotides. Two different types of precursors of dendritic cells can be found in humans. These are: (1)

Peripheral blood monocytes (pDC1); and (2) $CD4^+CD3^-CD11c^-$ plasmacytoid cells (pDC2). Peripheral blood monocytes (pDC1) give rise to immature myeloid DCs after culturing with GMCSF and IL-4. These immature cells give rise to mature myeloid dendritic cells (DC1) after stimulation with CD40 ligand (CD40L). When the mature myeloid dendritic cells are cultured with naive T helper cells, the naive T helper cells become TH1 type cells, and produce TH1 type cytokines, such as IFN-γ (Rissoan, et al., Science 283, 1183 (1999)).

$CD4^+CD3^-CD11c^-$ plasmacytoid cells give rise to immature lymphoid-type dendritic cell after culture with IL-3. These immature cells give rise to mature lymphoid-type dendritic cells after stimulation with CD40 ligand (CD40L). When the mature lymphoid-type dendritic cells are cultured with naive T helper cells, the naive T helper cells become T_H2 type cells which, in turn, produce T_H2-type cytokines, such as IL-4 (Rissoan, et al., Science 283, 1183 (1999)).

The above description relates to two broad scenarios. The first involves peripheral blood monocytes (pDC1) and their role, after stimulation, to promote the conversion of naive T-helper cells to T_H1 cells. The second scenario involves $CD4^+CD3^-CD11c^-$ plasmacytoid cells (pDC2) and their role, after stimulation, to promote the conversion of naive T-helper cells to T_H2 cells. The above two pathways communicate with each other in a manner mediated by IL-4 (product of T_H2 cells). With overproduction of IL-4, or during production of IL-4 during late stage in the immune response, this IL-4 inhibits the differentiation of $CD4^+CD3^-CD11c^-$ plasmacytoid cells (pDC2), and in this way feedback inhibits the production of TH2 type cells. With overproduction of IL-4, or during production of IL-4 during late stage in the immune response, the IL-4 stimulates the conversion of peripheral blood monocytes (pDC1) to immature myeloid dendritic cells, thus increasing the production of T_H1 type cells (Rissoan, et al., Science 283, 1183 (1999)).

EXAMPLE XIII. Natural interferon producing cells

The following commentary concerns some of the characteristics of a line of $CD4^+CD3^-CD11c^-$ plasmacytoid cells, which have been found to be a type of "natural interferon producting cell." The plasmacytoid morphology has been shown by Siegal, et al., Science 284, 1835 (1999)).

"Natural interferon producing cells" (IPC) are specialized leucocytes that are the major source of interferon-α in response to viruses, bacteria, and tumor cells. Another characteristic

of natural interferon producing cells (IPC) is that they express CD4 and Class II MHC. $CD4^+CD3^-CD11c^-$ type 2 cells have been identified as a type of IPC. $CD4^+CD3^-CD11c^-$ type 2 cells are dendritic cell precursors are cells that can respond to microbial challenge and, when challenged, can produce 200-1000 times more interferon than other blood cells after microbial challenge (Siegal, et al., Science 284, 1835(1999)). Production of interferon-α occurs in resonse to Sendai virus, heat-killed *S.aureus*, or UV-irradiated virus. The fact that the $CD4^+CD3^-CD11c^-$ type 2 cells produce interferon-α in the absence of other cells suggests that these cells are part of the *innate immune system* (Siegal, et al., Science 284, 1835(1999)).

EXAMPLE XIV. Subsets of precursors of human dendritic cells

The following cell lines were studied: (1) $CD4^+CD3^-CD11c^+$ immature dendritic cells. Note that these are $CD11c^+$; (2) $CD4^+CD3^-CD11c^-$plasmacytoid pre-dendritic cells (pDC2) (natural interferon producing cells). Note that these are $CD11c^-$; and (3) $CD14^+CD16^-$ monocytes (pDC1).

The above-mentioned cells are described by Rissoan, et al., Science 283, 1183 (1999) and by Siegal, et al., Science 284, 1835 (1999)).

The present study revealed the forms of Toll like receptors (TLRs) on the various cells lines, as well as the influences of various added factors on the expression of the various TLRs. These factors included: (1) GMCSF plus IL-4 on the TLRs; (2) CD40L; and (3) Interleukin-3 (IL-3).

$CD4^+CD3^-CD11c^+$ immature dendritic cells expressed high lveles of TLR1, 2, and 3, low levels of TLR 5, 6, 8, and 10, and undetectable levels of TLR 4, 7, and 9.

$CD4^+CD3^-CD11c^-$ plasmacytoid pre-dendritic cells (pDC2) expressed high levels of TLR 7 and 9, low levels of TLR 1, 6, adn 10, and undetectable levels of TLR 2, 3, 4, 5, and 8.

$CD14^+CD16^-$ monocytes (pDC1)expressed high levels of TLR 1, 2, 3, 5, and 8, low levels of TLR6, and undetectable levels of TLR 3, 7, 9, and 10.

The following concerns exposure of the cell types to various stimulants or factors. Where $CD14^+CD16^-$ monocytes (pDC1)are differentiated into immature dendritic cells by exposure to GMCSF plus IL-4, the initial high expression of TLR2 and TLR4 decreased dramatically, where further decline occurred with CD40L treatment. This decrease in TLR2 and

TLR4 expression is consistent with the functional switch of the dendritic cell lineage from a microbial antigen recognition to antigen presentation (presentation to naive T cells).

$CD4^+CD3^-CD11c^+$ immature dendritic cells express moderate levels of TLR2 and TLR4, where expression decreases with exposure to CD40L.

$CD4^+CD3^-CD11c^-$ plasmacytoid pre-dendritic cells (pDC2), which do not express TLR2 or TLR4 at any stages of maturation.

$CD4^+CD3^-CD11c^-$ plasmacytoid pre-dendritic cells (pDC2)express TLR7 and TLR9, where expression of these two receptors progressively decreases with stimulation by IL-3 (to provoke differentiation to immature dendritic cells) and by CD40L (to provoke further differentiation to mature lymphoid dendritic cells).

Responses to peptidoglycan, lipopolysaccharide, lipoteichoic acid, unmethylated CpG oligonucleotides, and poly I:C were studied. Peptidoglycan (TLR2 ligand) stimulated $CD14^+CD16^-$ monocytes (pDC1) to produce TNF-α and IL-6. Peptidoglycan stimulated $CD4^+CD3^-CD11c^+$ immature dendritic cells to produce TNF-α, and small amounts of IL-6 and IL-12. Peptidoglycan did not stimulate $CD4^+CD3^-CD11c^-$ plasmacytoid pre-dendritic cells (pDC2)to produce any of the cytokines tested.

Lipotechoic acid (LTA), another TLR2 ligand, was tested. Its effects on the three cell lines did not exactly parallel those of peptidoglycan. LTA stimulated the monocytes to produce TNF-A and IL-6, but did not stimulate the $CD4^+CD3^-CD11c^+$ immature dendritic cells to produce detectable levels of the cytokines tested. LTA did not stimulate the plasmacytoid pre-dendritic cells.

Lipopolysaccharide (LPS) is a ligand for TLR-4. LPS stimulated monocytes to produce TNF-α and IL-6. LPS stimulated $CD4^+CD3^-CD11c^+$ immature dendritic cells to produce small amounts of IL-12p75, in two out of four human cell donors. LPS did not stimulate the plasmacytoid pre-dendritic cells to produce any of the cytokines tested.

Unmethylated CpG oligonucleotide (AAC-30) is a ligand for TLR9. AAC-30 did not stimulate monocytes or $CD4^+CD3^-CD11c^+$ immature dendritic cells to produce IFN-α, but did stimulate plasmacytoid pre-dendritic cells to produce IFN-α.

Poly I:C did not stimulate monocytes, and did not stimulate plasmacytoid pre-dendritic cells, but did stimulate $CD4^+CD3^-CD11c^+$ immature dendritic cells to produce IFN-α and IL-

12p75. Although AAC-30 and poly I:C are both comprised on nucleic acid, they had dissimilar effects on the three cell lines tested.

EXAMPLE XV. Treatment of viral diseases and tumors

Interferon-α is used to treat a number of viral disease, including hepatitis B, hepatitis C, hepatitis D (Di Bisceglie, New Engl. J. Med. 330, 137 (1994); Hoofnagle and Di Bisceglie, New Engl. J. Med. 336, 347 (1997), and T-cell leukemia-lymphoma (Gill, et al., New Engl. J. Med. 332, 1744 (1995)). Interferon-α is also useful for treating multiple myeloma (Bataille and Harousseau, New Engl. J. Med. 336, 1657 (1997)) and chronic myeloid leukemia (Faderl, et al., New Engl. J. Med. 341, 164 (1999); Porter, et al., New Engl. J. Med. 330, 100 (1994)). Diseases and disease states that are responsive to treatment with interferon-α may be called interferon-α treatable conditions.

Activating antibodies (anti-TLR9) are contemplated, where these antibodies provoke plasmacytoid pre-dendritic cells to secrete interferon-α. The invention contemplates use of anti-TLR9 to provoke plasmacytoid pre-dendritic cells to secrete interferon-α for use in treating interferon-α responsive diseases, including those described above.

EXAMPLE XVI. Treatment of systemic lupus erythematosus by anti-TLR9 or by soluble TLR9

Systemic lupus erythematosus (SLE) is a disease involving elevated serum interferon-α. In SLE, complexes of anti-DNA (autoantibodies) and DNA are found in the bloodstream (Ronnblom and Alm, Trends in Immunol. 22, 427 (2001)). These complexes stimulate natural interferon-α producing cells, e.g., plasmacytoid pre-dendritic cells, where stimulation results in the secretion of interferon-α. This secreted interferon-α sustains the generation of more autoantibodies.

Antibodies to TLR9 are contemplated, where these antibodies are inactivating antibodies, and where the inactivating antibodies inhibit TLR9 and prevent TLR9 ligands from activating the cell to secrete interferon-α. Also contemplated is use of soluble versions of TLR9 to bind to anti-DNA/DNA complexes, thus preventing these complexes from activating TR9 (thus preventing the consequent secretion of interferon-α).

EXAMPLE XVII. Treatment of septic shock by an antibody to an TLR-4 or by soluble TLR-4

Serious infections may result in a system response to the infection called sepsis. When sepsis results in hypotension and organ dysfunction, it is called septic shock (Parrillo, New Engl. J. Med. 328, 1471 (1993)). Gram-positive organisms, fungi, and endotoxin-containing gram-negative organisms can initiate a series of events resulting in sepsis and septic shock. One feature of septic shock is decreased use of oxygen by various tissues of the body. Another feature is that many vascular beds are abnormally dilated, while others are abnormally constricted, resulting in maldistribution of blood flow (Parrillo, New Engl. J. Med. 328, 1471 (1993)).

Endotoxin is a lipopolysaccharide associated with cell membranes of gram negative microorganisms. Studies with experimental animals and with humans have shown that endotoxin causes septic shock (Parrillo, New Engl. J. Med. 328, 1471 (1993)). Endotoxin is a ligand for TLR4 (Kadowaki, et al., J. Exp. Med. (in press); Thomas, New Engl. J. Med. 342, 664 (2000); Tapping, et al., J. Immunol. 165, 5780 (2000); Supajatura, et al., J. Immunol. 167, 2250 (2001); Hoshino, et al., J. Immunol. 162, 3749 (1999)). Bacterial products have been found which are ligands for TLR2. These products, which may contribute to the pathology of septic shock, have not yet been identified (Tapping, et al., J. Immunol. 165, 5780 (2000)).

It is contemplated to use anti-TLR4 or soluble TLR4 for treating disease conditions such as sepsis, where the disease conditions involve interaction of bacterial, microbial, or fungal products with TLR-4.

EXAMPLE XVIII. Treatment of septic shock by an antibody to an TLR-2 or by soluble TLR-2

Gram positive organisms can cause sepsis, where the natural products identified as causative agents have been identified as peptidoglycan, and lipoteichoic acid (Schwandner, et al., J. Biol. Chem. 274, 17406 (1999)). Capsular polysaccharide of Streptococcus, a gram positive organism, is a cause of sepsis and neonatal meningitis in Japan (Kogan, et al., J. Biol. Chem. 271, 8786 (1996)). A number of natural products have been found to stimulate TLR2, including yeast cell walls, spirochetal lipoproteins, whole mycobacteria and mycobacterial lipoarabinomannan, whole gram positive bacteria, and gram positive bacterial lipoteichoic acid, and peptidoglycan (Schwandner, et al., J. Biol. Chem. 274, 17406 (1999); Tapping, et al., J. Immunol. 165, 5780 (2000)).

It is also contemplated to use anti-TLR2 or soluble TLR2 for treating disease conditions such as sepsis, where the disease conditions involve interaction of bacterial, microbial, or fungal products with TLR-2.

All citations herein are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled; and the invention is not to be limited by the specific embodiments that have been presented herein by way of example.